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Doctoral Thesis

IDENTIFICATION OF BIOMARKERS FOR THE ASSESSMENT OF FARM RUMINANT HEALTH STATUS

(VET/03)

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Abstract

The aim of this PhD thesis was to identify biomarkers for evaluating the health of farm ruminants and possible the quality of products. A rapid identification and quantification of acute phase proteins (APPs) can provide useful information on the health status of animal.

From a clinical perspective, the availability of a quick, and reliable, diagnostic marker specific for inflammatory diseases is of paramount importance.

APPs can be employed in the assessment of animal health and welfare both ante- and post-mortem as an aid to meat inspection.

This work can be divided into two principal studies: the first one focused on the localization of APPs in extra hepatic tissues; in particular we have analyzed the distribution of acute phase proteins in the bovine forestomachs and abomasums and then the expression of SAA and Hp RNA in bovine tissues (and evaluation of suitable reference genes). The second part was more focused on the search for specific inflammatory biomarkers; in turn, this part of the thesis can be sub-divided into 2 studies: *Escherichia coli* lipopolysaccharides and *Staphylococcus aureus* enterotoxin B relationship with inflammatory microRNAs in bovine monocytes and the development of a highly sensitive sandwich ELISA to quantify the goat and bovine AGP and SAA in serum.

Regarding the first part of our investigations we demonstrated the expression of the four APPs' mRNA. These results were confirmed by Western blot analysis followed by

localization through immunohistochemistry. We have also identified SAA and Hp's mRNA in all analyzed tissues in non pathological conditions.

In the second part of the thesis, we investigated the effect of *Escherichia coli* lipopolysaccharide (LPS) and *Staphylococcus aureus* enterotoxin B (SEB) on the expression of five miRNAs involved in the inflammatory response, including miR-9, miR-125b, miR-155, miR-146a and miR-223, in bovine $CD14^+$ cells (monocytes). We obtained an up-regulation both miR-155 and miR-146a when cells were stimulated with LPS and a down-regulation of miR-125b, miR-155 and miR-223 after SEB stimulation, suggesting miRNAs as potential inflammatory biomarkers.

The last part of the study was devoted to ELISA detection of APP. A precise and sensitive test to determine AGP content was developed as sandwich ELISA in serum of bovine and goats. A good analytical sensitivity was demonstrated. The test can detect a wide range of AGP concentrations. Yet, neither in goats nor in bovine species, the test managed to identify the animals with disease. Regarding SAA, a novel immunoassay to specifically detect SAA in ruminants' serum was developed, but the present results must still be regarded as preliminary.

1. Introduction

1.1 Acute Phase Reaction

Modern livestock systems are mainly focused on balancing animal health with animal welfare. Animal health can be defined as the absence of disease, determined by clinical examinations combined with different diagnostic tests. Clinical examinations, however, suffer from low reproducibility and cannot detect subclinical disorders, which are traditionally detected by pathology, microbiology or serology. Anyway, these techniques are not efficient enough at the farm and slaughterhouse level to detect animal health deficiencies *(Petersen et al., 2004)*. Animal welfare can be defined as the situation that comprises the state of an animal's body and mind, and the extent to which its nature is satisfied *(Hewson, 2003)*. The current importance of animal welfare in livestock production is not only focused in ethics, but in its direct influence on production performance and meat quality *(Gispert et al., 2000)*.

In mammals, a complex network of intercellular and intracellular signalling participates to the maintain of homeostasis after stimulus by recognizing non-self elements in the body, involving pro-inflammatory processes, including cytokines, cytokine modulators, and hormones associated to a number of systemic changes referred as the acute-phase response (APR). It is the innate, non-specific response preceding specific immune reactions. The acute phase reaction (APR) is a dynamic process, a prominent response of the organism to local or systemic disturbances in its homeostasis caused by infection, tissue injury, trauma or surgery, neoplastic growth or immunological disorders *(Gruys et al., 1999)*. Local inflammation is the major reaction of the body upon tissue injury caused

by infection. Inflammation is basically a protective response, the ultimate goal of which is to rid the organism of both initial cause of cell injury and the consequences of such injury. At the site of invasion by a microorganism or the place of tissue injury a number of responses of the tissue itself are initiated. Monocytes, platelets and endothelial cells participate in vascular inflammation that regulates the humoral innate immunity and participates in homeostatic processes by activating, for example, anti-inflammatory regulators. During repair the injured tissue is replaced by regeneration of native parenchymal cells, by filling the defect with fibroblastic tissue or by a combination of both. The inflammatory response occurs in the vascularised connective tissue, including plasma, circulating cells, blood vessels, cellular and extra-cellular constituents of connective tissue. If this delicate balance between inflammation and resolution becomes deregulated, inflammation can lead to disease.

At the site of invasion by micro-organisms and in the place of tissue injury, a number of responses of the tissue itself are initiated. These processes lead to release of pro-inflammatory cytokines and other inflammatory molecules (*van Miert, 1995)*. These cytokines, together with nitric oxide and glucocorticoids, trigger and modulate the systemic acute phase reaction which includes several phenomena such as fever, leukocytosis and hepatic over expression of proteins collectively called acute phase proteins (APPs), this last feature being also called the hepatic acute phase protein response *(Gruys et al., 1994; Heinrich et al., 1990; 1998; van Miert, 1995)*.

Historically, APR is assessed as an innate immune response. Nonetheless, several differences between degrees of intensity of the reaction have been detected. For example, bacterial infection usually leads to a painful systemic acute phase response *(Alsemgeest et al., 1994)*, due to the strong reaction of the mononuclear-phagocytic system's cells.

TNF-α and IL-1ß are induced in response to endotoxin *(Monshouwer et al., 1996a; 1996b; Schindler et al., 1990; Werling et al., 1996).* In viral infections, the APR is generally milder *(Höfner et al., 1994; Kimura et al., 1994; Nakayama et al., 1993).* The principal cytokines released by infected cells are primarily interferons (IFNs), especially IFNγ from mononuclear inflammatory cells, although TNF-α and IL- 1ß from tissue cells may be involved as well. In case of severe cellular damage, a full APR can be generated *(van Reeth et al., 1998).*

Inflammation may also develop due to non-infectious causes as in varieties of tumours. In cancer cytokines can induce APR without exogenous inflammatory stimuli. IL-6, for example, can be produced by a number of different cell types (such as keratinocytes, endothelial cells and fibroblasts) under the influence of circulating IL-1 and TNFα. This cytokine activation and release leads to high levels of IL-1, IL-6 and TNF-α in the blood *(Moshage, 1997; Gabay and Kushner, 1999).*

Not only pathogenic, but also, in a lesser extent, non-pathogenic microorganisms harbour the highly conserved non-self molecules which are critical for their survival or for their pathogenicity. During infectious processes cells, including macrophages respond to exogenous danger signals induced by the pathogens associated molecular patterns (PAMPs) or Microbe-associated molecular patterns (MAMPs) that are not found as a part of eukaryotic cells. This response is amplified by endogenous mediators released and by co-factors or concomitant stressful events, and molecular mechanisms involved in the vicious circle destruction-reconstruction of vessels and tissues, act through injury-associated signals known as Damage-Associated Molecular Patterns (DAMPS or Alarmins) and acute phase proteins. Some DAMPs engage TLRs to induce and amplify the inflammatory response. TLR2 and TLR4 signalling have been shown to mediate NF-

κB activation initiated by HMGB1 *(Park et al., 2006)*. Other molecules, such as S100A8 and S100A9, participate to migration and cytoskeletal rearrangement. Different signalling pathways are involved that may cross-talk at several levels, but all culminate in the activation of NF-κB. Cell damage or activation of phagocytes triggers their release into the extracellular space where they become danger signals that activate immune cells and vascular endothelium. S100A8 and S100A9 seem to interact with RAGE4 and TLRs *(Vogl et al., 2007)*; the nuclear, high mobility group box-1 (HMGB1) protein that is not characterized by having pro-inflammatory activity but it binds LPS, DNA or IL-1ß and induces signalling pathways leading to NF-κB activation thereby enhancing inflammatory pathways; and Serum amyloid A (SAA) released by necrotic cells are the major DAMPs increased in serum of several inflammatory diseases, including cancer, sepsis, atherosclerosis, and arthritis. Several receptors appear to mediate the effect of SAA, including FPRL1, RAGE, TLR2 and TLR4. The downstream signalling pathways triggered by SAA include ERK and p38 activation induces chemotactic for neutrophils and the production of pro-inflammatory cytokines and NO *(He et al., 2009*, *Sandri et al., 2008)*. DAMPs activate innate immune response through pattern recognition receptors (PRRs) *(Bianchi, 2007)* such as receptor for advanced glycation end products (RAGE) found in endothelial cells and macrophages and activate MAP kinase-dependent inflammation upon interaction with one of the following factors HMGB1, S100 proteins and ß-amyloids *(van Beijnum et al., 2008)*.

Among PAMPs, Gram-negative and Gram-positive bacteria respectively express at their surface LPS and peptidoglycan as well as lipotechoic acid. In addition, molecules found in microorganisms include mannose (almost absent in humans), bacterial un-methylated

CpG DNA, bacterial flagellin, the amino acid N-formyl-methionine found in bacterial proteins, double-stranded and single-stranded RNA from viruses, and glucans, mannans, and zymosan from fungal cell walls. More than 1.000 recognition elements have been identified and designated by soluble pattern-recognition receptors.

1.2 Regulation of Acute Phase Reaction

The inflammatory response is coordinated by a large range of mediators that form complex regulatory networks. The activity of these mediators can be figured as a network, in which each molecule can act singly, in combinations or in sequence, thus amplifying the inflammatory response and modulating its evolution. Many mediators have been identify and are summarized in table 1.1.

Origin		Mediators	Source
cellular	Preformed mediators in secretory granules	Histamine	Mast cells, basophil, platelets
		Serotonin	platelets
		Lysosomal enzymes	Neutrophils, macrophages
	Newly synthesized	Prostaglandins	All leukocytes, platelets, endothelial cells
		Leukotrienes	All leukocytes
		Platelet-activating factor	All leukocytes, endothelial cells
		Activated oxigen species	All leukocytes
		Nitric oxide	Macrophages
		Cytokines	Macrophages, lymphocytes, endothelial cells
Liver→plasma	Factor XII (Hageman factor) activation	Kinin system (bradykinin)	
		Coagulation-fibrinolysis system	
	Complement activation	C_{3a}	
		C_{5a}	
		C_{3b}	
		C_{5b-9}	

Table 1.1. Chemical mediators of inflammation *(Robbins, 1999)*.

From a clinical point of view, the acute phase proteins may be classified in "major APP", when their serum concentration increases more than 5 folds, or "minor APP", if their concentration increases less than 5 folds. The intracellular mechanism for the induction of positive APPs in hepatocytes by cytokines has been reported elsewhere *(Jensen and Whitehead, 1998)*.

Cytokines are a group of proteins acting as intracellular and intercellular signalling molecules. The role of cytokines during inflammation is both initiation and fine-tuning of the whole process: some cytokines initiate and amplify the response, others sustain or attenuate it, and some of them cause it to resolve.

Cytokines can orchestrate a wide array of changes which occurs at sites distant from those of original inflammatory reaction. This wide-range systemic response is called "acute phase reaction", or, more properly, "systemic reaction to inflammation". More properly because a systemic reaction may occur and, usually, it does, not only during acute inflammatory events, but also after, or due to, chronic ones.

Generally speaking at least 15 different low molecular weight cytokines are known to be secreted by activated leukocytes (interleukins) and other cells. Three main groups of cytokines corresponding to effect pathways can be categorized *(van Miert, 1995)*:

(1) cytokines that primarily act as positive or negative growth factors for a variety of cells (IL-2, IL-3, IL-4, IL-7, IL-10, IL-11, IL-12 and granulocyte- macrophage colony stimulating factor),

(2) cytokines with pro-inflammatory properties (TNF-α/ß, IL-1α/ß, IL-6, IFN-α/γ, IL-8, and macrophage inhibitory protein-1), and

(3) factors with anti-inflammatory activity (IL-1 receptor antagonists, soluble IL-1 receptors, TNF-α binding protein and IL-1 binding protein).

The proinflammatory cytokines including interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1ß) are the major mediators of acute phase reaction for acute phase protein (APP) synthesis in the liver *(Nakagawa-Tosa et al., 1995; Alsemgeest et al., 1996; Yoshioka et al., 2002)*. These cytokines are released mainly by macrophages but also by other cells in response to various external or internal stimuli. For example, IL-6 can be synthesized by Kupffer cells and keratinocytes *(Heinrich et al., 1990)*, in the pituitary *(Abraham and Minton, 1997)* or in the mucosal epithelium *(Pritts et al., 2002)*.

Glucocorticoids, which are synthesized through the HPA-axis (hypothalamic pituitary adrenal-axis), act to suppress pro-inflammatory cytokines and up-regulate anti-inflammatory cytokines *(Bethin et al., 2000)*. Thus the HPA-axis contributes to the modulation of APP synthesis. Conversely, cytokines modulate glucocorticoid production by the HPA-axis. For example, IL-6 stimulates the HPA-axis and induces augmented adrenal function, which in turn suppresses cytokine production by negative feedback *(Bethin et al., 2000)*. Induction of APR and regulation of APP synthesis has been shown in a diagram in fig. 1.1.

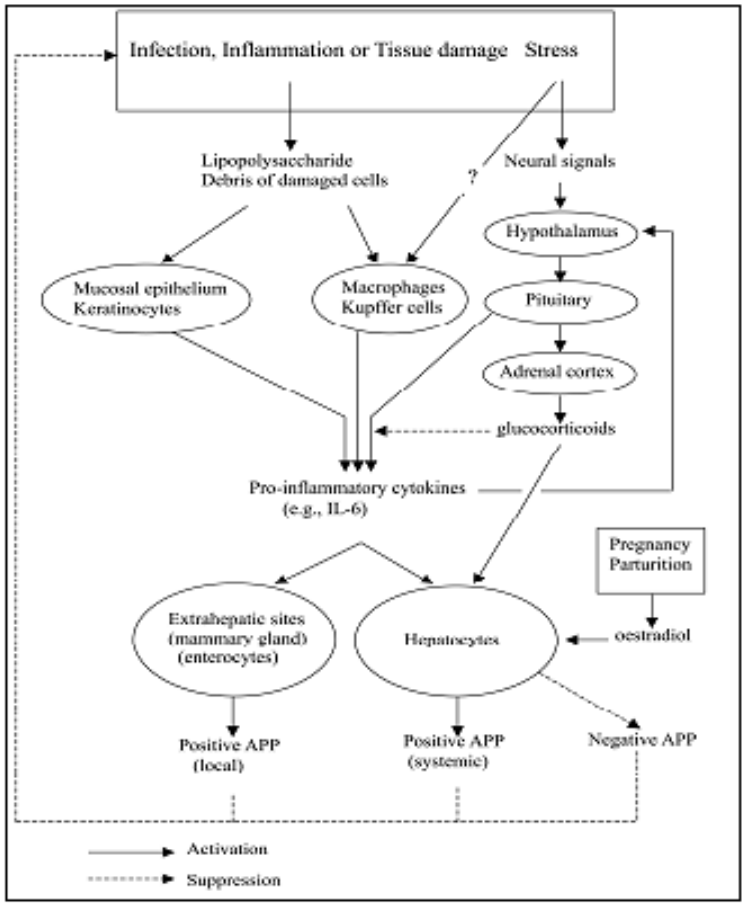

Figure 1.1: Induction and regulation network of acute phase protein (APP) synthesis in animals subjected to internal or external challenge *(Murata et al., 2004)*.

It is widely accepted that, in humans and experimental animals, physical and psychological stress elevates plasma IL-6 and APPs levels *(Deak et al., 1997; Nukina et al., 2001)*. APPs are plasma proteins that modify their concentration following infection, inflammation, trauma or stress *(Murata et al., 2004)*. The circulating concentrations of these proteins can provide an objective unit of measurement of the health status of an animal and are increasingly being used as markers for animal health and welfare in farm animals such as pigs or cattle. APPs are mainly of hepatic origin and their synthesis is regulated by pro-inflammatory cytokines, hormone peptides that act as mediators between the damaged tissues and the liver *(Baumann and Gauldie, 1994)*. Pro-inflammatory cytokines also exert its activity in different targets, leading to a systemic reaction that includes profound metabolic changes. Appetite diminishes and muscle catabolism is accelerated, resulting in weight loss. Thus the APPs assay can identify herds where poor hygiene, lack of surveillance, poor vaccination responses or other factors have lead to immunological stress and a reduction of feed conversion. Measuring APPs concentration will be valuable to assess the health status of new groups of animals entering to the farm, as well as for final inspection of slaughter cattle or pigs, improving surveillance programs.

There is also evidence in cattle that physical stress can induce APPs *(Murata and Miyamoto, 1993; Alsemgeest et al., 1995)*.

Piñeiro et al., (2007) found that pig road transport can increase APPs in serum, supporting the concept that non-inflammatory, psychophysical stress can induce a discernible APPs response in healthy domestic animals. Although the mechanism of

APPs induction in response to stress is yet to be elucidated, *Murata (2007)* proposed a hypothesis that could explain the nature of the stress-APPs linkage.

A successful acute inflammatory response results in the elimination of the infectious agents followed by a resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophages *(Serhan & Savill, 2005)*. The switch in lipid mediators from pro-inflammatory prostaglandins to lipoxins, which are anti-inflammatory, is crucial for the transition from inflammation to resolution. Lipoxins inhibit the recruitment of neutrophils and, instead, promote the recruitment of monocytes, which remove dead cells and initiate tissue remodelling *(Serhan & Savill, 2005)*. Resolvins and protectins, which constitute another class of lipid mediator, as well as transforming growth factor-ß and growth factors produced by macrophages, also have a crucial role in the resolution of inflammation, including the initiation of tissue repair *(Serhan & Savill, 2005; Serhan, 2007)*.

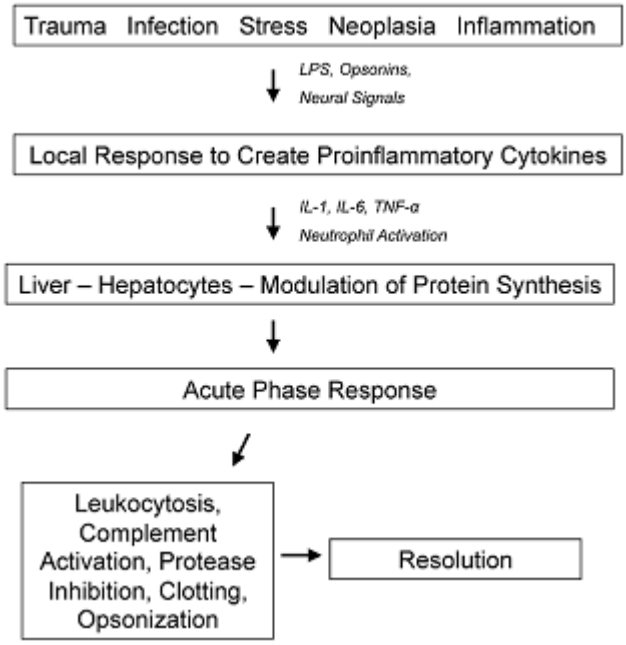

Figure 1.2. The acute phase response *(Cray, 2009)*.

If the injurious agent cannot be quickly eliminated, the result may be chronic inflammation. Chronic inflammation is a prolonged process where active inflammation, tissue destruction and attempt at healing may all proceeding simultaneously. Considerable progress has been made in understanding the cellular and molecular events that are involved in the acute inflammatory response. On the contrary, the events that lead to local chronic inflammation, particularly in chronic infections and autoimmune diseases, are still partially understood. Much less is known, however, about the causes and mechanisms of systemic chronic inflammation, which occurs in a wide variety of diseases, such as, for example, type 2 diabetes and cardiovascular diseases. Therefore, given that inflammation not only is beneficial but also can be damaging if prolonged, it is not surprising that the entire process is tightly regulated.

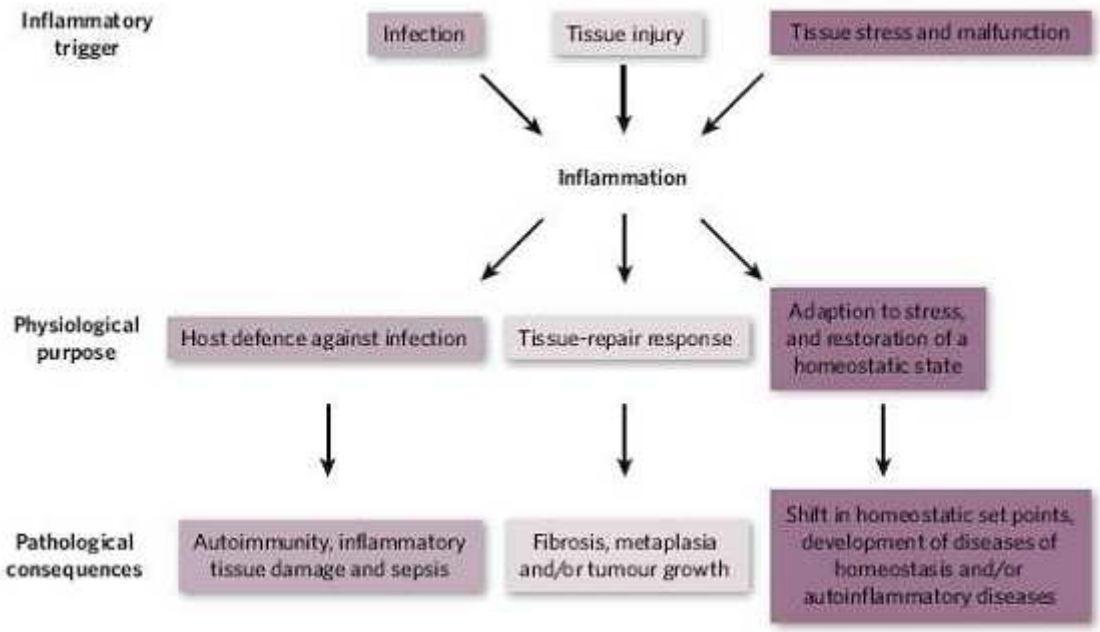

Figure 1.3. Causes, and physiological and pathological outcomes, of inflammation. Depending on the trigger, the inflammatory response has a different physiological purpose and pathological consequences. Of the three possible initiating stimuli, only infection induced inflammation is coupled with the induction of an immune response *(Medzhitov, 2008)*.

1.3 Innate Immunity and Acute Phase Reaction

Innate immunity is the most universal and the most rapidly acting type of immunity *(Beutler, 2004)*. After tissue injury, trauma, or infection, the vertebrate host responds immediately with a variety of non adaptive physiological changes, collectively known as the innate immune response. This response encompasses diverse countermeasures, such as the complement system, natural killer cells, the acute phase response, and cytokine response *(Flint et al., 2000)*.

Innate Immunity comprises the cells and mechanisms that defend the host from infection by other organisms, in a non-specific manner. This means that the cells of the innate system recognize and respond to pathogens in a generic way, but unlike the adaptive immune system, it does not confer long-lasting or protective immunity to the host *(Bruce et al., 2002)*. Innate immune systems provide immediate defence against infection. The principal components of the innate immune system include cellular and humoral elements. In order to recognize invading organisms, cells participating in the innate immune response such as macrophages, monocytes, granulocytes and dendritic cells, need and share receptors that are able to distinguish self from non-self *(Medzhitov and Janeway, 2002)*. The intracellular signaling pathways are activated after pathogen detection and leads to cytokine production *(Akira et al., 2001)*. Cytokines, in turn, contribute to the many features of the inflammatory response including: (1) the febrile response; (2) the induction of hepatic synthesis of acute phase proteins; (3) leukocyte recruitment; and (4) changes in vascular permeability, tone, and activation *(Dinarello, 1996; Thijs et al., 1996)*. Detection of infectious pathogens is mediated by the expression of evolutionarily conserved pattern recognition receptors that are capable of recognizing common bacterial motifs shared by diverse pathogens *(Akira et al., 2001)*. The toll-like receptors (TLR) are a highly conserved family of pattern recognition receptors involved in pathogen detection. Bacterial LPS contains motifs that are recognized by TLR-4. Presentation of LPS to this receptor is mediated by the accessory molecules CD14 and LPS-binding protein (LBP). TLR pairs, including TLR-1 and 2 and TLR-2 and 6, recognize lipopeptides, whereas, individual TLR's, such as TLR-5 and TLR-9, recognize flagellin and un-methylated CpG DNA *(Akira, 2003; Adamo et al., 2004*; *Greene et al., 2005)*. Acute Phase Proteins may modulate the immune response in one way or another.

For example, LBP binds LPS and assists in its further detection; C-reactive protein, a pentraxin, binds to the capsule of streptococci and may signal their presence *(Beutler, 2004)*. As previously described, the acute phase response is dependent upon cytokines, which elicit responses in tissues that may be far removed from the site of infection (the brain, where fever is concerned, or the liver, where the synthesis of acute-phase reactants takes place).

1.4 The function of acute phase proteins

Acute phase proteins are believed to play major roles in several aspects of the systemic reaction to inflammation, including the opsonization of several pathogens, the scavenging of potentially toxic substances and the overall regulation of different stages of inflammation. The acute phase response is thought to be part of a general defense-response towards tissue injury *(Gauldie et al., 1989)*. It is generally accepted that the acute phase response induced fever and slow-wave sleep is in some way beneficial to an organism under physical stress *(Kluger et al., 1975)*. A number of the common APPs (i.e. those that are acute phase proteins in most species) are likely to participate directly in the protection of the host. During the acute phase response, the serum concentration of the APP changes dramatically. The APP can be produced by both hepatocytes and peripheral tissues, and can be classified according to their concentration in positive APP, if they increase, or negative APP if they decrease *(Petersen et al., 2004)*. Morley and Kushner (1982) defines APPs' as plasma proteins which increase or decrease by at least 25 percent during inflammatory disorders. In some diseases, C-reactive protein may increase more than 1000 times. The plasma concentrations of the APPs are related to the

severity of the disorder and the extent of tissue damage in the affected animal and therefore quantification of their concentration can provide diagnostic and prognostic information if proper timing of sampling is assured. Remarkably, some proteins that are down regulated during acute phase in serum, are on the contrary up-regulated in extra-hepatic tissues. This is the case of albumin which acts as a positive APP in mammary gland during mastitis *(Shamay et al., 2005)*. Many advances in monitoring the APPs response in animals for clinical and experimental purposes have been achieved in the last two decades *(Gruys et al., 1994)*. For example, researchers have established various quantitative APPs assays, thereby recognizing the species-related difference of the APPs behaviour on some key proteins. There is also growing interest in elucidating the mechanisms of the response and standardizing APPs assays, both of which could provide benefits to veterinary medicine *(Eckersall et al., 1999a)*.

In veterinary medicine distinct positive APPs from some species do not behave in the same way in other species (table 1.2); for example Serum Amyloid P-component (SAP) is an APP in the mouse, but not in man, and CRP reacts as APP in several monogastric species, but not very well in ruminants *(Gruys et al., 1994)*. Transferrin, which is a negative APP of most mammalian species, reacts as positive APP in chicken *(Hallquist and Klasing, 1994; Tohjo et al., 1995)*. CRP, SAA, Hp and other APPs, have been described as useful for assessing health in human patients *(Blackburn, 1994; Ferard et al., 2002)* and in various animals *(Gruys et al., 1994; Petersen et al., 2004; Toussaint, 2000; Toussaint et al., 1997)*, including also marine mammalians *(Duffy et al., 1996; Funke et al., 1997)*.

CRP, a major APP in humans, became the biomarker of choose in human medicine for systemic inflammation and infection monitoring. The utilisation of APPs for monitoring

inflammatory activity has been recently adopted in veterinary clinical chemistry, mainly due to a lack of available commercial tests for routine analysis, a lack of quality control materials for laboratories to be confident of providing valid results and a lack of knowledge on the interpretation of the data. The potential uses of APPs vary according to species *(Eckersall, 2004)*.

	Chicken	Dog	Horse	Pig	Ruminants
AGP	▲	▲	▲-●	▲	▲
Cp	▲	▲	●	●	●
CRP	NC	■	▲	▲	▲
Fb	●	▲	▲-●	▲-●	▲
Hp	NC	▲	▲	▲	■
SAA	▲	▲	■	▲	▲-■

Table 1.2. Classification of APP in domestic animals: ■, major (10–100-fold increase in response to stimuli); ▲, moderate (2–10-fold increase); ●, minor (<2-fold increase); and NC, not confirmed *(Murata et al., 2004)*.

Cytokines activity and the corresponding acute phase reaction elicited by each of them have been published for ruminants, horse, pig and several other species *(Gruys et al., 1994)*. APPs are used with conditions varying from mastitis in cows *(Eckersall et al., 2001)*, to cattle with tropical theileriosis *(Glass et al., 2003)*, to cats with feline infectious peritonitis (FIP) *(Duthie et al., 1997)*, to dogs with multicentric lymphoma *(Nielsen et al., 2007)*, and to horses with influenza *(Hultén et al., 1999)*. It is also possible that subclinical inflammation could be identified by APPs assays *(Eckersall et al., 2006)*.

In the field, increased APPs levels can be used to identify animals with sub-clinical disease, allowing not only early treatment of infection, but also isolation of infected

animal, hence preventing the spread of disease within intensive systems *(Petersen et al., 2004)*. Animals suffering from disease also suffer from associated starvation and therefore negative energy balance as muscles are catabolised to provide amino acids for APPs production and as energy source *(Gruys et al., 2005)*. APPs could have potential use not only in identifying animals suffering from severe stress, but also in general welfare management, i.e. high APPs levels indicate underlying problems whether these are hygiene or disease related *(Murata, 2007)*.

In this thesis we are focusing on four major/moderate APPs in ruminant and these proteins are: Serum Amyloid A (SAA), Haptoglobin (Hp), lipopolysaccharide binding protein (LBP) and α1-acid glycoprotein (AGP) sharing similar functional activity. Therefore all of them fulfil two different classes of functions: they are binding proteins and they can regulate innate immunity reactions *(Ceciliani et al., 2012)*. The functions of each of these four APPS are therefore detailed hereby.

1.5 Serum amyloid A

Serum amyloid A (SAA) proteins are a family of apolipoproteins, is a non-glycosylated protein with a molecular weight ranging between 11 and 14 KDa, depending on species (*Benditt & Eriksen, 1977; Nakayama et al., 1993; Gruys et al., 1994)*, associated with high-density lipoprotein (HDL) in plasma. Different isoforms of SAA are expressed constitutively (constitutive SAAs) at different levels or in response to inflammatory stimuli (acute phase SAAs). The conservation of these proteins throughout invertebrates and vertebrates suggests SAAs play a highly essential role in all animals *(Manley et al., 2006)*.

Serum amyloid A is so named due to its involvement in reactive amyloidosis although from studies on mice it may be that only one isoform (SAA-2 see below) is predominantly found in amyloid deposits *(Bowman, 1993)*. In fact, an increase of SAA concentration in serum and tissues is believed to be the prerequisite for the precipitation of the insoluble form of SAA, the AA form in amyloidotic fibrils. Three genes and a pseudo-gene encoding SAA have been found in the human genome *(Steel et al., 1993)*. They have been classified in two groups: the acute phase SAA (A-SAA) and the constitutive SAA (C-SAA). Three genes belong to A-SAA group, namely SAA-1, SAA-2 and SAA-3. A fourth gene encodes for the constitutive SAA-4. The two major circulating forms include SAA1 and SAA2, both of them 104 residues long. They are produced by liver during the acute phase after induction by pro-inflammatory cytokines, such as IL-6 and TNF-α *(Uhlar and Whitehead, 1999)*. The SAA3 is synthesized mainly in extra-hepatic tissues. In bovine, a mammary associated SAA3 (M-SAA3) has been isolated and characterized *(McDonald et al., 2001)*. At the moment the complete biological function of this protein is not yet elucidated, but researchers have to identify three different functions: binding of cholesterol, immunomodulatory activity and opsonisation. Figure 1.4 presents a synthesis of the major functions of SAA.

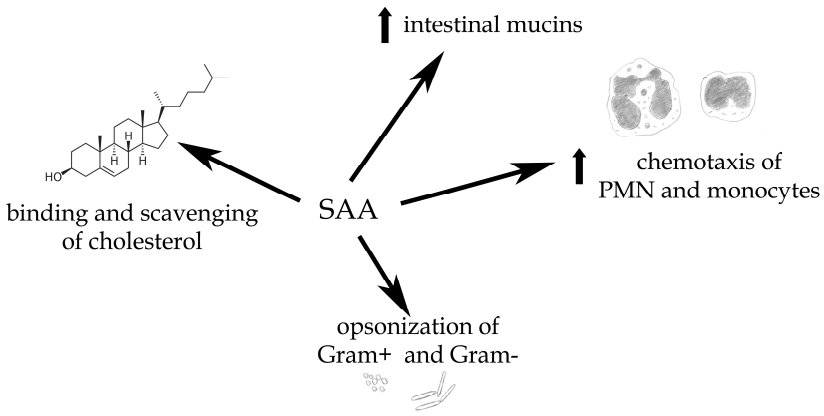

Figure 1.4. Major functions of SAA *(Ceciliani et al., 2012)*.

One major role proposed for HDL is to reverse cholesterol transport, which is removing cholesterol from peripheral tissues to the liver for catabolism and secretion. This is vital, since the liver is the major site of cholesterol degradation and removal. HDL's role in reverse cholesterol transport is supported indirectly by the observation that HDL concentrations are negatively associated with the risk of coronary heart disease from atherosclerosis *(Lacko, 1994)*. Under non-inflammatory conditions, HDL contains very low levels of SAA *(Rosenthal et al., 1976)*. Once inflammation occurs, SAA levels increase dramatically. SAA associated HDL_3 seems to facilitate the uptake and removal of cholesterol from monocytes/macrophages at the inflammatory site *(Banka et al., 1995)*. Therefore, one important function of SAA is to scavenge the cholesterol from dying cells, thus preventing its accumulation in atherosclerotic plaques *(Manley et al., 2006)*.

The physiological role of SAA in host defense during inflammation is not well understood, but various effects have been reported. These include detoxification of endotoxin, inhibition of lymphocyte and endothelial cell proliferation, inhibition of

platelet aggregation, and inhibition of T lymphocyte adhesion to extracellular matrix 37 proteins *(Urieli-Shoval et al., 2000)*.

A direct effect of SAA on immune cell has been reported, and is been saw that this protein acts as a chemoattractant and mediates the migration, adhesion and tissue infiltration of monocytes and neutrophils *(Badolato et al.,1994)*. It may also play a role in down-regulation of the inflammatory process by inhibiting myeloperoxidase release and directed migration of phagocytes *(Gatt et al., 1998)*. Further activity also includes up-regulating IL-8 expression *(He et al., 2003)*. The chemotactic activity of SAA is regulated through FPRL1 receptor, which reacts with formyl-L-methionyl-L-peptide (fMLP) *(Su et al., 1999)*. Since fMLP does not induce chemotaxis in bovine PMN *(Targowski et al., 1986)*, the chemotactic-modulating activity of SAA in cattle has still to be demonstrated.

Intestinal epithelial cells can release SAA upon stimulation by the pro-inflammatory mediators TNF-α, IL-6 and IL-1ß *(Vreugdenhil et al., 1999)*. The protein may be involved in the local defence mechanism of the gut to endotoxins challenge. The secretion of mammary-associated SAA has been detected in normal bovine, equine and ovine colostrums and mammary epithelium, suggesting a role related to newborn adaptation to extrauterine life and possibly mammary tissue remodeling *(McDonald et al., 2001; Larson et al., 2005 and Weber et al., 2006)*.

The antibacterial activity of bovine SAA is probably wider, since it is directed toward both $Gram^+$ and $Gram^-$ bacteria *(Molenaar et al., 2009)*. It must be said that most of the experiments on bovine SAA have been carried out on the mammary gland M-SAA3. Therefore, it is possible that circulating forms of SAA have different behaviors. The

finding that lipoteichoic acid (LTA), the Gram$^+$ -associated pathogen associated molecular pattern (PAMP), stimulates the expression ofM-SAA3 by mammary gland epithelial cell *(Weber et al., 2006)* suggests that, at least in this species, SAA is involved in mammary gland defense against both Gram$^+$ and Gram$^-$ pathogens. The finding that bovine SAA can be found along forestomachs mucosa *(Dilda et al., 2012)* supports this hypothesis.

1.5.1 Hepatic and extra-hepatic production of SAA

The hepatic acute phase response is induced by a wide range of mediators, including IL-1, IL-6, TNF-α, leukemia inhibitory factor (LIF), transforming growth factor ß (TGF ß), interferon ɣ (INF ɣ), IL-11, oncostatin, ciliary neurotrophic factor (CNF) and retinoic acid, whereas others such as insulin and okadaic acid have inhibitory effects *(Steel et al., 1993)*. Glucocorticoids have dual actions: they enhance the effects of other mediators on hepatocytes *(Steel et al., 1993)* and at the same time down-regulate the production of, for example, TNF-α and IL-1 by macrophages and polymorphonuclear cells (PMN). The above mediators act by binding to their respective receptors on the cell surface. After binding they are internalized and/or transmit signals into the cells to effect a number of different intracellular regulatory mechanisms, activation and de novo synthesis of transcription factors and changes in processing and stability of RNA, translation efficiency and protein secretion. The principal cytokines involved in the induction of A-SAA are IL-1, TNF-α and IL-6. Other cytokines that may be involved either directly or indirectly in A-SAA induction include IL-2, interferon-ɣ (IFN-ɣ) and ciliary neutrophic factor *(Numerof et al., 1992*; *Fantuzzi et al., 1995)*. Glucocorticoids, which are also

released during inflammation, have been shown to enhance cytokine-induced A-SAA expression. Although the liver is the major site of APP synthesis, the extra-hepatic tissue/cellular expression of a wide range of APPs has been documented *(Colten, 1992)*. In most mammalian species, SAA1 and SAA2 are predominantly synthesized in the liver while SAA3 is the main isoform expressed at extra-hepatic sites *(Husby et al., 1994)*. Bovine mammary gland epithelial cells (MAC-T) have been shown to produce a mammary associated SAA3 (M-SAA3) under normal physiological conditions *(Uhlar et al., 1999)*. Stimulation of MAC-T cells with LPS and prolactin revealed an increased expression of M-SAA3 while other SAA transcripts were not detected *(Larson et al., 2005)*. In another study these authors used *S. aureus* lipoteichoic acid for stimulating MAC-T cells and the predominant isoform expressed was SAA3 *(Weber et al., 2006)*. In cell culture SAA mRNA expression has been shown in normal mammary tissue *(Molenaar et al., 2009; Berg et al., 2011)*. Expression is low in epithelial cells with a histologically apparent lactation phenotype and high in ductal epithelial cells, vesicle engorged alveoli and inflamed alveoli *(Molenaar et al., 2009)*. The M-SAA3 peptide showed antibacterial activity against *E. coli*, *Streptococcus uberis* and *P. aeruginosa (Molenaar et al., 2009)*. Experimental infection with *S. aureus* resulted in region-specific SAA3 changes *(Whelehan et al., 2011)*. Mammary SAA3 expression was increased in alveolar, ductal and gland cistern tissues 48 h post challenge *(Whelehan et al., 2011)*. Berg et al. *(Berg et al., 2011)* reported the expression of SAA mRNA in the ovary (<1% of hepatic expression) and uterus (>1% of hepatic expression). Epithelial and stromal cells from healthy uteri were able to synthesize SAA *(Whelehan et al., 2011)*. In uterine biopsies, SAA3 mRNA was increased in early postpartum cows in comparison to late postpartum cows and SAA reflected the severity of inflammation *(Chapwanya et al.,*

2009). Surprisingly high expression rates of SAA mRNA were also shown in bovine forestomachs: in rumen, omasum and reticulum 78, 7 and 168-fold higher mRNA concentrations than in liver were recorded, respectively *(Dilda et al., 2012)*. A significant protein expression of SAA was limited to the abomasums *(Dilda et al., 2012)*.

In relation to the function of APPs in fat tissue, there are several studies indicating that they are involved in the low-grade inflammation associated with obesity which is characterized by adipocyte hypertrophy and macrophage infiltration into adipose tissue *(Wellen et al., 2003)*. For SAA3, the inducible adipocyte-derived isoform of SAA, a role in monocyte recruitment and adhesion acting in concert with hyaluronan but independently from monocyte chemoattractant protein-1 (MCP-1) has been documented *(Han et al., 2007)*. Direct or indirect relationships between SAA2 and PPAR-γ may also exist since activation of PPAR-γ in mice reduced SAA3 expression *(Han et al., 2007)*. In ruminant adipose tissue, there is evidence at the mRNA level for the expression of SAA in bovine adipose tissue *(Berg et al., 2011)* and differences in SAA mRNA abundance between different visceral and subcutaneous depots have already been established *(Saremi et al., 2012)*. Examination of APPs response in cows with abomasal displacement has revealed that SAA was increased in cows with either left abomasal displacement, right abomasal displacement or abomasal volvulus *(Guzelbektes et al., 2010)*.

1.5.2 Bovine SAA

Measurements of serum concentration have demonstrated that SAA is a major acute phase protein in cattle, but its diagnostic value as an indicator of inflammation in clinical investigation has not been determined. In cattle, an increased SAA serum and plasma concentration has been found following experimentally induced and naturally occurring inflammation as well as experimental and natural infection. SAA has been shown to reflect the severity of mastitis; cows with clinical mastitis had almost 100-fold levels of SAA in serum and milk compared with healthy cows *(Eckersall et al., 2001)*. A total of seven different SAA isoforms were characterized in the serum of cows with chronic inflammation or amyloidosis and in healthy cows. The isoforms are distinguishable using the isoelectric point (pI) in 2-dimensional electrophoresis. Isoforms of pI 5.2 and 8.6 are the only two identified in amyloidotic sample *(Takahashi et al., 1989)*. SAA1 and SAA2 are expressed constitutively by liver and SAA3 is expressed locally and also present in milk. This last isoform is also called Milk Amylod A (MAA) because it was found in milk from dairy cows *(Eckersall et al., 2001; McDonald et al., 2001)*. The sequence data obtained from genomic walking revealed a four-exon, three-intron organization for bovine MAA, typical of the other sequenced SAA genes *(Ulhar and Whitehead, 1999)*. Exon 1 contained a 53 bp 5'-untraslated region (UTR), downstream and adjacent to this first exon was a 556 bp intron. The second exon (92 bp) contains the beginning of the coding sequence for the MAA gene precursor protein and exon 3 (139 bp) encoded the next 46 aminoacids. Exon 4 encoded the last 55 aminoacids. The splice junction for all the three introns conformed to the highly conserved 5'-GT/AG-3' consensus sequence for splice donor/acceptor sites at the two ends of an intron. A polyadenilation signal (AATAAA) was located 97 nucleotides downstream to the STOP

codon and was 26 nucleotides upstream of a GT-rich region, typically found 11 to 30 nuclotides after this site *(Larson et al., 2005).*

SAA is elevated more by acute rather than chronic inflammatory conditions *(Horadagoda et al., 1999)* and is also raised following experimental infection with *Mannheimia haemolytica*, and bovine respiratory syncytial virus. There have been recent advances in characterizing the APP response in specific infectious and inflammatory disease and in the use of the APP as a biomarker for innate immunity in a variety of research studies.

Cattle infected with foot and mouth disease virus are known to exhibit a Hp response *(Höfner et al., 1994)* and this was confirmed in a further study of experimentally induced disease, where both Hp, SAA and type 1 interferon were increased at onset of viraemia and clinical disease *(Stenfeldt et al., 2011).* However the APP were not able to differentiate between carrier and non-carrier status after the concentrations returned to baseline. In a comparison of the response of N'Dama and Boran cattle to experimental *Trypanosoma congolense* infection, SAA increased by 14 days post infection with greater increases found in the trypanotolerant N'Dama breed than in the Boran which may be related to the greater susceptibility of the latter to Trypanosome infection *(Meade et al., 2009).* The acute phase proteins, Hp, SAA and LBP were found to be sensitive markers of bovine respiratory disease in natural cases of bovine respiratory syncytial virus infection of calves *(Orro et al., 2011).* Higher concentrations of APPs at later stages of the infection were associated with lower specific anti-virus IgG1 production suggesting that such calves had an enhanced inflammatory response to a secondary infection. In a further study of naturally occurring and persistent bovine viral diarrhea virus infection, cattle were found to have significantly higher serum concentrations of SAA than healthy cattle

from the same herds although the increases were moderate being 3.6-fold *(Ulutas et al., 2011)*. A study of lameness in cattle in relation to APPs in serum has shown that cattle with severe sole ulcers and white line abscesses had significantly elevated concentrations of SAA *(Kujala et al., 2010)*. The effect of transport stress on cattle has shown that after 4 to 6 h of transport in solitary tie stalls the serum concentrations of SAA were significantly increased *(Lomborg et al., 2008)* indicating that it could serve as marker of stress in cattle.

1.6 α_1 Acid glycoprotein (AGP) or orosomucoid (ORM)

Alpha-1-acid glycoprotein (AGP) was concomitantly first described in 1950 by Schmid (1950 and 1953) *(Schmid 1950; Schmid 1953)* and Weimer (1950) *(Weimer et al., 1950)*, and turned out to be a very unusual protein with a very low pI of 2.8-3.8. Due to its very high carbohydrate content of more than 40% it is also called orosomucoid. AGP is one of the major acute phase proteins and has been extensively studied for many years, mainly due to its drug and many other molecules like steroid hormones. AGP three dimensional structure clearly resembling the conformation of a transport protein, as rich as it is in ß-sheets. In normal physiological condition AGP can bind more than 300 different molecules and drugs *(Israili et al., 2001)*. The binding and delivery function of AGP is remarkable, given the background that AGP may markedly increase its concentration during acute phase, thus becoming one of the most abundant proteins in serum *(Sheldon et al., 2001; Eckersall et al., 2001)*. The molecules that are bound and transported by AGP could be classified in several groups including inflammatory mediators, bacterial derived molecules and drugs. AGP can interact with several inflammatory-mediators, such as heparin and serotonin *(Schmid et al., 1973)*, platelet activating factor *(Mcnamara et al., 1986)*, histamine *(Chachaj et al., 1980)* and plasminogen activator inhibitor type I (PAI-1) *(Boncela et al., 2001)*.

AGP is constitutively expressed by hepatocytes; it is found mostly in blood, in varying concentration, depending on species (table 4). The physiological plasma concentration ranges from 0.3 to 0.5 mg/ml, depending on the species, but can increase up to 2-5 times with systemic inflammation *(Hochepied et al., 2003)*. One of the most interesting

features of AGP is that its concentration in plasma not only rises during inflammation, but also undergoes structural modification of its oligosaccharide moiety, resulting in a change of both degree of branching and fucosylation. In vivo studies in rats showed that increases in the amount of diantennary glycans on the surface of AGP can be induced by IL-6 and glucocorticoids *(Mackiewicz et al., 1991)*.

Species	Concentration in plasma (mg/ml)	Increase during Acute Phase Reaction (Petersen et al., 2004)
Human	0.5-1 (Kremer *et al.*, 1988)	I
Bovine	0.2-0.45 (Horadagoda *et al.*, 1999)	I
Pig	0.3-0.56 (Eckersall *et al.*, 1996)	0/I
Dog	0.05-0.8 (Kuribayashi *et al.*, 2003)	I
Cat	0.1-0.48 (Duthie *et al.*, 1997)	I
Mouse	0.2-0.4 (Kopf *et al.*, 1994)	I
Rat	0.1-0.2 (Arnold and Meyerson, 1990)	II

0: No change. I: between 100% and 10 times increase. II: more than 10 times increase

Table 1.4. Serum α1 Acid glycoprotein concentration in different species *(Ceciliani and Pocacqua, 2007)*.

Bovine AGP is a 219 residue long glycoprotein, with a molecular weight of 20.5 kDa. The primary structure of bovine AGP also includes five glycosylation and eight potential phosphorylation sites *(Ceciliani et al., 2005; Balaguer and Neusüess, 2006)* (determined using http://www.cbs.dtu.dk/services/NetPhos/). From a structural perspective AGP is a lipocalin. It belongs therefore to that group of proteins that can bind and transport small hydrophobic molecules *(Flower et al., 2000)*. AGP has been further classified in a subset of lipocalins, the so-called immunocalins, a subfamily of proteins that may also immunomodulate the inflammatory reaction *(Lögdberg and Wester, 2000)*. Two kinds of receptors have been identified in monocyte-derived macrophages (MDM) *(Atemezem et al., 2001)*, monocytes and granulocytes *(Shiyan and Bovin, 1997)*, one high-affinity (Kd

values= 1.6×10^{-9} M) and one low-affinity receptor (Kd values= 4.9×10^{-6} M). At least one of these receptors was identified as the receptor for CCR5 chemokines. AGP features at least four biological activities, which are apparently very different from one another, but are actually strongly related. AGP may act as plasma transport protein and immunomodulate the inflammatory response. AGP may further protect the organism against bacteria, and acts as chaperone (Fig. 1.5).

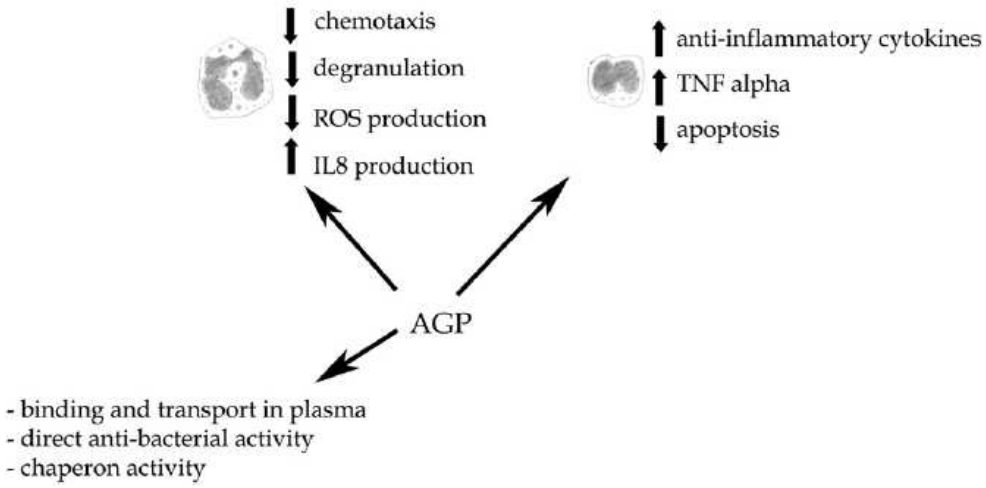

Fig.1.5: the biological functions of α1 Acid glycoprotein *(Ceciliani et al., 2012).*

AGP can modulate a wide number of immune functions, mainly in white blood cells.

Several studies focusing on immunomodulatory activity of AGP have been carried out in cattle. Bovine AGP is a powerful anti-inflammatory protein, and modulates all the stages of the monocytes and PMN attack sequence, including chemotaxis, reactive oxygen species (ROS) production and degranulation. When utilized at high concentrations, like those found in serum during systemic reaction to inflammation, bovine AGP inhibits the migration of monocytes. The chemotactic-modulating activity was shown to be dependent on CD18 down-regulation, thus suggesting that AGP

interferes with the adhesion of the cells on endothelium membrane *(Lecchi et al., 2008)*. The killing activity of PMN can be modulated as well. Bovine AGP down-regulates PMN extracellular release of ROS, while still maintaining the capability to kill $Gram^+$ bacteria such as *S. aureus (Rinaldi et al., 2008)*. An IL-8 up-regulatory activity has also been demonstrated. Further immunomodulatory activity of bovine AGP includes the down-regulation of secondary granule exocytosis *(Miranda-Ribera et al., 2010)*. Since both human *(Theilgaard-Mönch et al., 2005)* and bovine *(Rahman et al., 2008)* AGP are stored in secondary granules, and are released from the cells after activation, it can be hypothesized that one of the functions of AGP is to fulfill a paracrine and endocrine activity on PMN that have been attracted to the inflammatory focus, in order to dampen their very aggressive attitude toward surrounding tissues. It is worth noting that AGP activity is very selective. It is cell specific, since for example AGP can reduce chemotaxis of monocytes, but not PMN *(Lecchi et al., 2008)*, and cell-compartment specific, since it down-regulates degranulation of secondary granules of PMN, but it is not effective on primary ones *(Miranda-Ribera et al., 2010)*.

An acute phase concentration of AGP reduces the apoptosis of bovine monocytes (*Ceciliani et al., 2007)*. Monocytes contribute to the resolution of inflammation by removing the inflammatory cells, such as exhausted PMN. Therefore, by increasing their lifespan, AGP can also prolong the anti-inflammatory activity of monocytes in the inflammatory focus. This apoptosis-inhibitory activity has also been shown for human AGP *(de Vries et al., 2004; Van Molle et al., 1999)*.

Immunomodulatory activity of bovine AGP is strongly related to its glycan moiety: both exocytosis down-regulating activity of neutrophils and apoptosis inhibiting activity on monocytes are suppressed by the removal of sialic acid terminal residues exposed on the

protein surface *(Miranda-Ribera et al., 2010; Ceciliani et al., 2007a)*. This glycan-dependent immunomodulatory activity is remarkable, since the glycosylation of AGP is strongly dependent on its physiological and pathological status *(Ceciliani et al., 2007)*.

As for other APPs, a chaperone like activity has been envisaged for AGP. By using a thermal aggregation model, Zsila demonstrated that human AGP reduced the clustering of both enzymatic and non-enzymatic proteins *(Zsila, 2010)*.

The extra-hepatic expression has been reported in several other tissues, including human and bovine mammary epithelial cells *(Gendler et al., 1982; Ceciliani et al., 2005)*, stimulated alveolar macrophages *(Fournier et al., 1999)*, prostate *(Poland et al., 2002)*, pig's nasal mucosa *(Guiraudie et al., 2003)*. White blood cells, such as cultured human monocytes *(Shibata et al., 1984, Nakamura et al., 1993)* and resting and activated polymorphonuclear leukocytes *(Gahmberg and Andersson, 1978)*, can also express AGP. Human granulocytes synthesize a specific glycoform of AGP, which is stored in the specific azurophylic granules, and is rapidly released together with lactoferrin following activation. AGP's expression in a myeloid cell line can be induced by members of the C/EBP (CCAAT/enhancer-binding protein) family *(Poland et al., 2005; Theilgaard-Mönch et al., 2005)*.

In the uterus, moderate levels of AGP mRNA was detected *(Lecchi et al., 2009)*, whereas in the ovary AGP mRNA was detected at low levels *(Lecchi et al., 2009)*.

AGP mRNA expression in both abomasums and forestomachs was demonstrated, albeit at much lower concentrations than in liver *(Dilda et al., 2012)*.

AGP is reported to be adipose derived APP *(Castriota et al., 2007)*. AGP mRNA and protein is present in adipose tissue from dairy cattle *(Rahman, personal communication)*.

Ceciliani and colleagues *(Ceciliani et al., 2007b)* found AGP in bovine milk produced mainly from alveolar epithelium had different molecular weight that of plasma AGP. Major expression of AGP was confirmed in the salivary glands and spleen, whereas minor expression was detected in all other tissues sampled, including lung, lymph nodes, kidney and tongue *(Lecchi et al., 2009)*. Endothelial lining of both blood vessels and lymphatics also have the capability to produce AGP in normal physiological condition *(Lecchi et al., 2009; Sörensson et al., 1999)*.

1.6.1 Immunomodulatory Activities of AGP

For its anti-neutrophil and anti-complement activities AGP has been considered as a natural anti-inflammatory and immunomodulatory agent *(Williams et al., 1997)*. Indeed, researchers confirmed AGP's role in vitro and in vivo as an immunomodulating molecule. The most important activity of AGP on macrophages is probably the induction of expression of molecules that antagonize the activity of IL-1ß and TNF-α, such as IL-1 receptor antagonist and soluble TNF receptor. While acting as an anti-inflammatory molecule, it has also been reported that AGP may also induce monocytes to express pro-inflammatory cytokines, such as IL-6 and IL-12. Interestingly, low concentrations of LPS synergistically act with AGP in induction of both IL-1 receptor antagonist and IL-1ß *(Tilg et al., 1993)*. Moreover, human monocytes stimulated with AGP produce the other pro-inflammatory cytokine, TNF-α, together with Tissue Factor *(Su and Yeh, 1996)*.

There are some proteins such as C3, soluble CD14 and IgG which can bind AGP and these proteins can also induce AGP for enhanced production of TNF-α *(Su et al., 1999)*. It has been further demonstrated that the expression of TNF-α is due to activation of a tyrosine kinase dependent pathway. It is possible that, since CD14, C3 and IgG are expressed on the surface of monocytes, the binding of these proteins enhances AGP's interaction with monocytes (*Hochepied et al., 2003)*.

AGP can inhibit the chemotactic response of neutrophils challenged with formylmethionyl-leucyl-phenylalanine (fMLP) at physiological concentration *(Lainé et al., 1990)*. At low doses (0.3 mg/ml), AGP promotes the aggregation of neutrophils, but at higher doses (>0.5 mg/ml) AGP inhibits this aggregation *(Lainé et al., 1990; Costello et al., 1984)*.

AGP plays several important roles that involve binding/ transport of small molecules and modulation of the inflammatory response. Circular dichroism investigations *(Nishi et al., 2002 and 2004)* revealed that the binding of AGP to membrane results in a structural change from an original, prevalently ß-sheet, structure to a prevalently α-helix structure, that causes a local release of ligand concomitant with the conformational changes. Besides, the binding of progesterone with AGP induces the transformation of a α-helix to an anti-parallel ß-sheet *(Kopecký et al., 2003)*. The following figure (Fig. 1.9) shows a possible mechanism of action of AGP: the binding and transport of molecules depends on the ß-sheet conformation, and the interaction with a still unknown receptor is followed by a modification of the affinity, that causes a local discharge of the ligand.

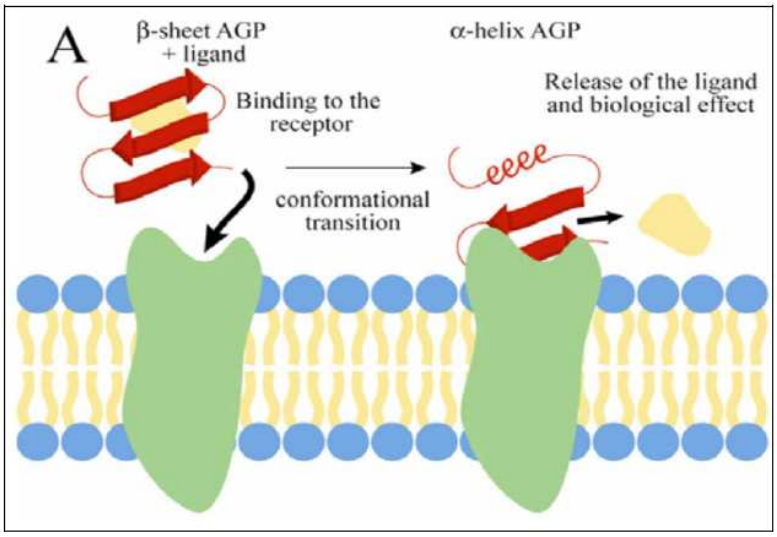

Figure 1.9: AGP binds and transports molecules on the site of inflammation. The interaction with the receptor or the cellular membrane of the target induces a conformational transition from a ß-sheet rich structure to a α-helix rich structure that causes a local release of the ligand *(Ceciliani and Pocacqua, 2007).*

From many studies it is evident that the various interactions of AGP with leukocytes are dependent on modifications in glycosylation such as branching and sialylation degree. An increase in fucosylation, that corresponds to an increase in sLe^X structures on AGP surface, has been shown to ameliorate neutrophil- mediated damage in organs such as lung and intestines and the glycoforms lacking sLe^X groups are clearly less active *(Williams et al., 1997).*

Experimental data clearly shows that the prevalent function of hyperfucosylated AGP is probably antinflammatory. This "ameliorating" biological function may be exerted in several ways, the most important probably being the direct interaction of sLe^X residue with E-selectins of the activated endothelium, thus reducing the sLe^X mediated rolling of granulocytes. This effect is likely increased by a second wave of granulocyte-derived

AGP, which is strongly fucosylated and locally produced. This hypothesis has been supported by the demonstration that different glycoforms of AGP expressed in rheumatoid arthritis can inhibit *in vitro* the interaction between sLe^X and E-selectin, which is the first step in the cell adhesion process *(Jørgensen et al., 1998)*. Figure 1.10 shows a possible model of the inhibition of sLeX rich AGP's isoforms on neutrophil rolling.

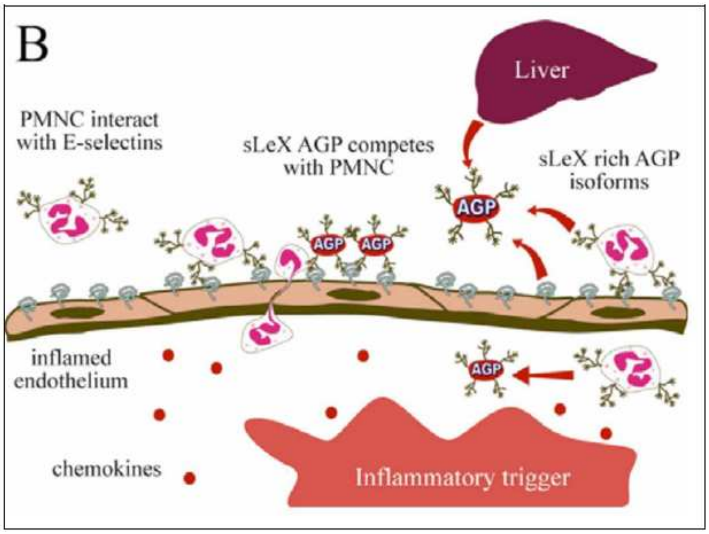

Figure 1.10: The inhibition of granulocytes extravasation by AGP expressed during inflammatory reaction. During normal inflammatory reaction the E-selectins are expressed over the surface of inflamed endothelial cells due to induction by chemokines and other inflammatory cytokines. E-selectins interact with sLe^X on the surface of granulocytes (PMNC), causing the cells to roll on the endothelium, adhere and extravasate toward the site of inflammation, following the gradient of concentration of chemotactic factors. Inflammatory, i.e. sLe^X rich, AGP expressed during systemic reaction by liver, but also by endothelium and activated granulocytes, interacts with E-selectins, thus competing with PMNC *(Ceciliani and Pocacqua, 2007)*.

1.7 Haptoglobin

Haptoglobin (Hp) is an acute phase plasma protein with haemoglobin-binding capacity and is characterized by a molecular heterogeneity showing three major phenotypes: Hp 1-1, Hp 2-2, and the heterozygous Hp 2-1. This polymorphism arises from variant α-chains (α1 and α2), since the ß-chain is identical in all haptoglobin types. This variation in a chain composition gives rise to differences in molecular mass, Hp 1-1 being a smaller protein (86 kDa) whereas Hp 2-1 (165 kDa) and Hp 2-2 (200 kDa) are polymerized forms *(Bowman, 1993a; Langlois and Delanghe, 1996 and Dobryszyck, 1997)*. Haptoglobin was identified as a hemoglobin binding protein in 1938 *(Polonovski and Jayle, 1938)*.

Bovine Hp is composed of two 20-kDa peptides (α chain) and two 35-kDa peptides (ß chain) linked by disulfide bonds *(Morimatsu et al., 1991)*. Purified native Hp has a molecular mass of 1000-2000 kDa, the stable unit being a α2-ß2 tetramer. This protein is not present in normal bovine serum. Large and heterogeneous molecular sizes with different degrees of polymerisation have also been reported *(Morimatsu et al., 1992)*. In cattle, plasma haptoglobin also exists as polymers in association with albumin *(Eckersall and Conner, 1990)*. The ß-chain of human Hp has four N-glycosylation sites at Asn 184, 207, 211 and 241 *(Nakano et al., 2008)*. In cows, only two N-linked glycan chains have been identified at Asn 286 and 316.

Interestingly, most of the activities of Hp are related to the formation of a Hp-Hemoglobin (Hb) complex, not to the purified Hp. Haptoglobin has long been identified as the principal scavenger of free hemoglobin in blood. Further biological activities, mostly derived from studies of Hp in non-ruminant species also include the regulation of

innate immunity reactions in white blood cells, direct bacteriostatic effect and a chaperone activity (Fig. 1.11).

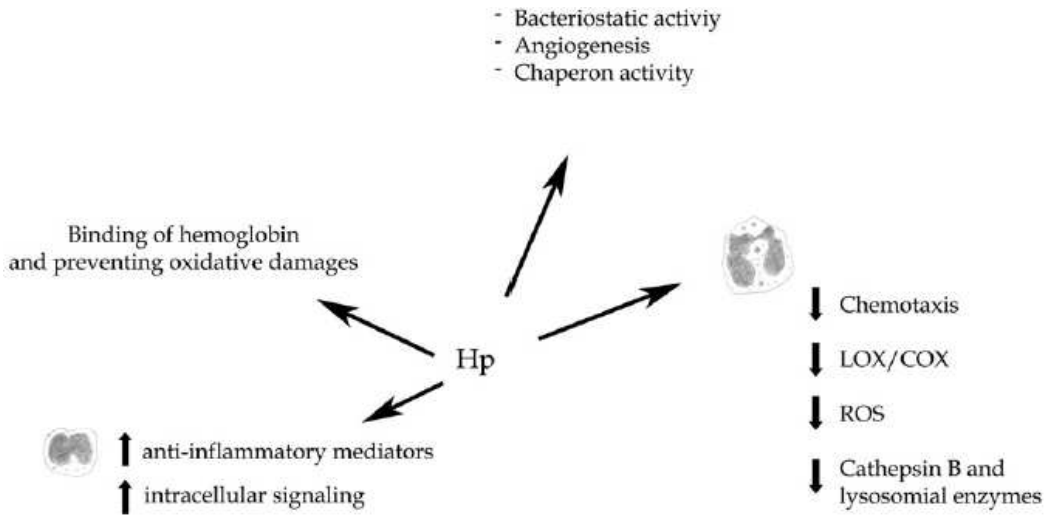

Figure 1.11: The biological functions of haptoglobin *(Ceciliani et al.,2012).*

Haptoglobin (Hp) has been described as one of the major acute phase proteins in cattle *(Eckersall and Conner, 1988).* Elevated concentrations of Hp in blood as well as in milk were demonstrated during the course of mastitis *(Eckersall et al., 2001; Grönlund et al., 2003; Hiss et al., 2004).* Haptoglobin mRNA expression was shown in bovine circulating leukocytes *(Thielen et al., 2005).* Thielen and colleagues *(Thielen et al., 2007)*, also found that Hp mRNA was expressed in bovine mammary epithelium and they confirmed it by *in-situ* hybridization. In 1995 Friedrichs and collaborators *(Friedrichs et al., 1995)* found that Hp expression was increased six fold in adipose tissues after stimulation with LPS in mouse. Extra-hepatic expression of Hp has been described in kidney, spleen, thymus, heart, brain, lung, testis, and epididymis of mice after intraperitoneal injection of LPS

(Kalmovarin et al., 1991). In 2005 was observed *(Li et al., 2005)* that, both keratinocytes and Langehans cells in skin of human, can express and produce this protein.

The bovine reproductive tract has been found to be a novel location for Hp expression in normal physiological conditions at first and the potential involvement of Hp in modulating reproductive function has been hypothesized *(Lavery et al., 2004).* The protein was detected in follicular fluid of water buffaloes *(Bergamo et al., 1995)* and in the ovary and oviduct of dairy cows in luteal and non-luteal phases of the oestrus cycle *(Lavery et al., 2003).* It was hypothesized that the protein contributes to ovarian follicular development and oviductal function *(Lavery et al., 2003).*

Oviductal Hp mRNA is expressed constitutively at low levels and elevated Hp mRNA expression during the peri-oestrus phase is limited to the liver. Local Hp expression in the endometrium has also been studied in normal physiological conditions. Haptoglobin mRNA is expressed in the endometrium of apparently healthy uteri; however, no differences could be observed with regard to the oestrus cycle *(Fischer et al., 2010).* During the postpartum period Hp is expressed at low levels reaching a peak at day 17 post partum *(Gabler et al., 2010).* This finding is in accordance with a previous study *(Chapwanya et al., 2009)* in which early post partum cows (two weeks post partum) showed higher Hp mRNA compared to late post partum cows (9 weeks post partum).

Hp mRNA expression was demonstrated in abomasums and forestomachs, albeit at much lower concentrations than in liver *(Dilda et al., 2012).* On the contrary, high protein expression was observed in all forestomachs and in the abomasums *(Dilda et al., 2012).* The forestomachs function as fermentation chambers in which polymeric plant carbohydrates are digested and metabolized to short chain fatty acids that are mostly

locally absorbed. The epithelia comprise a very large surface that is continuously exposed to microbes and their products and may thus have a specific role in immune surveillance albeit a secretory function is not documented *(Ceciliani et al., 2012)*.

There is increasing evidence that obesity in humans is associated with low-level inflammation. From studies in obese humans and obese animal models, adipose tissue is known to produce and to secrete numerous hormones, chemokines, cytokines and also APPs. Hp is one of the main acute phase protein that has been documented be expressed in human and /or rodents adipocytes *(Friedrichs et al., 1995)*. Based on the associations between body fat and some APPs and on the evidence of Hp mRNA and protein in biopsies from fat as well as the release of Hp from adipose explants from obese subjects, Hp was proposed as adiposity marker in humans *(Chiellini et al., 2004)*. The expression of Hp in adipose tissue is regulated by cytokines but is also connected with metabolism: peroxisome proliferator-activated receptor (PPAR)-γ, a transcription factor that plays a pivotal role in lipid biosynthesis, inflammation and insulin sensitivity *(Tontonoz and Spiegelman, 2008)*, is able to bind to the Hp gene promoter to arrest Hp gene transcription *(Vernochet et al., 2010)*. Hp has been detected in different subcutaneous and visceral depots from dairy cattle: both the mRNA and the protein are detectable in bovine adipose tissues and bovine adipocytes differentiating from primary pre-adipocytes are at least one source of Hp expression in fat *(Saremi et al., 2010; Saremi et al., 2012a)*. When characterizing the serum concentrations of Hp throughout lactation, we observed increasing values around parturition, that were also paralleled by the Hp mRNA abundance in liver and subcutaneous as well as visceral adipose tissue *(Saremi et al., 2012a)*. When comparing the Hp mRNA expression from liver with adipose tissues, a significant contribution of Hp from adipose tissue seems improbable, since the mRNA

abundance in the latter tissue was several orders of magnitude lower than that of liver; indeed, we found no association between Hp serum concentrations and body fat content in dairy cows *(Saremi et al., 2012a)*. This implies that at least for the range of body fat addressed in these studies, Hp is not an adiposity marker as proposed in humans.

1.7.1 Haptoglobin binds hemoglobin and prevents oxidative damages

The primary function of Hp is to prevent the loss of iron by the formation of very stable complexes with free hemoglobin in the blood *(Allison, 1958; Keene and Jandl, 1965; Laurell and Nymann, 1957)*. During intravascular hemolysis, Hp forms complexes that cannot pass through the glomerular filter *(Allison and Rees, 1957)* due to their large size. Each Hp molecule contains four binding sites, two for each Hb α-ß dimer *(Nagel and Gibson, 1971)*. Once formed, the complex is stable (Kd of 10-15) and is swiftly removed by the circulation by the monocyte/macrophage CD163 Hp-Hb receptor expressed on Kupffer cells in the liver *(Graversen et al., 2002)*. The half-life of the Hp-Hb complex during its clearance by CD163 ranges from 20 to 50 min *(Asleh et al., 2006; Kato, 2009)*. By binding Hb, Hp fulfills an additional anti-oxidant role of iron stabilization *(Lim et al., 1998)*, resulting in a reduction of oxidative damages to Hb itself *(Buehler et al., 2009)*, to albumin, since the free exchange of heme from Hb and albumin is inhibited *(Bunn and Jandl, 1968)*, lipids *(Melamed-Frank et al., 2001)* and eventually to the tissues, kidney in particular. It must be said that, in addition to its oxidative toxicity, free Hb can also bind nitric oxide in a fast and irreversible way, scavenging NO and limiting its bioavailability *(Rother et al., 2005)*.

1.7.2 The anti-inflammatory role of haptoglobin

Hp activity during the innate immunity response is basically anti-inflammatory, notwithstanding its involvement in the recruitment of neutrophils in the inflammatory focus via interaction with CD11b-CD18 *(El Ghmati et al., 1996)*.

The binding of the complex Hp-Hb to CD163 of monocytes/macrophages results in the up-regulation of anti-inflammatory mediators, such as inducible heme oxygenase-1 (HO-1) and IL-10 release *(Schaer et al., 2006; Philippidis et al., 2004)*, thus activating an anti-inflammatory response. The precise immunomodulatory activity of the Hb-Hp complex binding to cell surface CD163 is still elusive, since the previously described anti-inflammatory effects overlap with other apparently pro-inflammatory effects, such as the triggering of intracellular signaling cascade resulting in Ca^{2+} mobilization *(Ceciliani et al., 2012)*.

Haptoglobin interacts directly with neutrophils' surface through two distinct Hp binding sites, one with a Ka of 1.9×10^{7} M (1.3×10^{5} binding sites/cell) and the other with a Ka of 3.1×10^{6}/M (2.6×10^{5} binding sites/cell). Neutrophil activity can be down-regulated by inhibiting both lipoxygenase and cycloxygenase *(Saeed et al., 2007)*. Other reported anti-inflammatory activities of neutrophils include the inhibition of the respiratory burst *(Oh et al., 1990)* and of cathepisn B and lysosomal protease activity *(Snellman and Sylven, 1967)*, eventually protecting surrounding tissues by damages induced by neutrophil degranulation. Further immunomodulatory activities of Hp include the suppression of T cell proliferation through the inhibition of Th2 cytokine pattern release *(Arredouani et al., 2005)*. Hp has an inhibitory effect on granulocyte chemotaxis, phagocytosis and bactericidal activity *(Rossbacher et al., 1999)*. Hp may inhibit mast cell proliferation *(El-*

Ghmati et al., 2002), prevent spontaneous maturation of epidermal Langerhans cells (the antigen presenting cells of the skin) *(Xie et al., 2000)*.

1.7.3 Bacteriostatic effect

Hp is believed to have a bacteriostatic effect by restricting the availability of iron necessary for bacterial growth *(Allison, 1958; Bullen, 1981; Eaton et al., 1982)*. For example, human Hp was shown to inhibit the growth of *Streptococcus pyogenes in vitro (Delanghe et al., 1998)*. Hp binds free haemoglobin, which is toxic and proinflammatory *(Wagener et al., 2001)*, in the plasma and reduces the oxidative damage associated with haemolysis *(Yang et al., 2003)*.

This effect can be of course overcome by bacteria that can utilize hemoglobin even when it is complex to Hp, such as pathogens equipped with iron-acquisition systems *(Lewis and Dyer, 1995; Zakaria-Meehan et al., 1988)*.

1.7.4 Angiogenesis and chaperone activity

Purified human Hp has been shown to stimulate angiogenesis in a dose-dependent manner on umbilical vein endothelial cells *(Cid et al., 1993)*. Finally, the binding capability of Hp extends to a variety of other proteins, including citrate synthase, glutathione-S-transferase, lysozyme and ovotransferrin, inhibiting their stress-induced or heat-induced precipitation *(Yerbury et al., 2005)*. This protective effective is very

specific, and suggests that Hp also fulfills an important role as an extracellular chaperone.

1.8 Lipopolysaccharide binding protein (LBP): sentinel against bacteria

Lipopolysaccharide binding protein (LBP) was initially discovered as a 58-60 kDa acute phase reactant in rabbit serum *(Tobias et al. 1986)*. The protein is mainly synthesized by hepatocytes as 50 kDa secretory class 1 acute phase protein and released as 60 kDa protein after glycosilation which is especially involved in the recognition of pathogenic bacteria *(Schumann et al. 1990; Ramadori et al., 1990)*. LBP is one of the key members of the innate immune response against bacteria.

Based on primary structure similarities and conserved gene structure, LBP can be classified within a small family of lipid transfer/LPS-binding proteins, which include also bactericidal permeability increasing protein (BPI), cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PTP), involved in host defense *(Bingle and Craven, 2004)*. The main acknowledged function of LBP is to sense bacterial infection and transmit the signal to the membrane surface of the defensive cells by transferring LPS to membrane bound $CD14^{+}$. In addition to this function, LBP can also interact with other bacterial compounds, modulating the biological activity of immune cells. The functions of LBP are presented in Fig. 1.12.

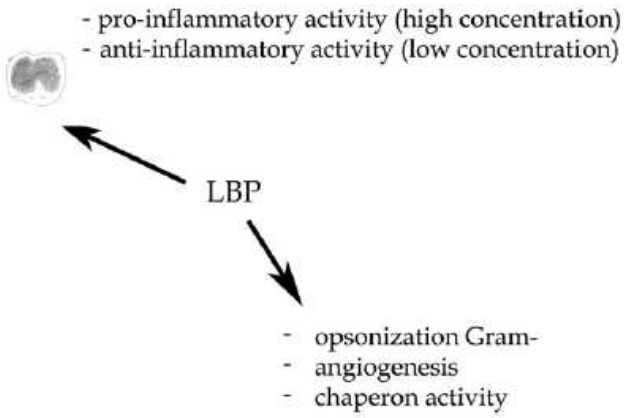

Fig. 1.12: The biological functions of lipopolysaccharide binding protein *(Ceciliani et al., 2012).*

The normal concentration of LBP in human serum is 5-15 µg/l and it increases during the acute phase response up to 30 fold. During the acute phase response, IL-1 and IL-6 synergize in inducing LBP synthesis, leading to an increase of LBP serum concentrations *(Schumann et al., 1996; Opal et al., 1999; Froon et al., 1995; Gallay et al., 1994).* Liver is the principal organ to synthesize acute phase proteins. It has recently been discovered that epithelial cells of the intestines the lungs, and skin *in vitro*, human gingival tissues both normal and infected as well as small muscle cells of the lung arteries, heart muscle cells and renal cells may represent additional sources of LBP *(Vreugdenhil et al., 1999; Dentener et al., 2000; Su et al., 1994*; *Ren et al., 2004).* Other studies showed that this protein is localized in secretory granules of paneth cells of small intestine in mouse *(Hansen et al., 2009).* Experiments in a model of experimental meningitis suggest a source of LBP within the central nervous system *(Weber et al., 2003).*

Mammary tissue from healthy bovines exhibits high expression of LBP in the teat cistern and the parenchyma *(Rahman et al., 2010).* With regard to pathological conditions (*E. coli* infection), LBP expression is increased in lobulo-alveolar regions but not in the teat cistern or the gland cistern *(Rinaldi et al., 2010).*

In the uterus of healthy cattle moderate levels of LBP mRNA was detected *(Lecchi et al., 2009; Rahman et al., 2010).*

mRNA of LBP has been documented in bovine lung and the pulmonary expression of the protein was immunohistochemically localized to resident macrophages and type II pneumocytes *(Rahman et al., 2010).*

In the digestive tract of bovine high LBP mRNA expression has been demonstrated, in particular was observed in the large salivary glands, i.e. in the parotid and in the submandibular gland about 50 and 30 fold higher mRNA abundance values were recorded than in liver; together with the evidence provided for the presence of protein by immunohistochemistry, an immunomodulatory function of LBP in the oral cavity is thus indicated *(Dilda et al., 2012; Rahman et al., 2010).* Surprisingly high expression rates of LBP mRNA were also shown in bovine forestomachs: in rumen, omasum and reticulum 19, 22 and 73 fold higher LBP mRNA concentrations than in liver were observed. In contrast, the mRNA expression in the abomasum that functionally corresponds to the stomach of monogastric species, was well below the hepatic mRNA abundance for LBP *(Rahman et al., 2010).*

LBP mRNA and protein is present in adipose tissue from dairy cattle *(Rahman personal communication).*

Lipopolysaccharide binding protein *(Schroedl et al., 2001)* has also been determined in milk (and serum) in dairy cows with mastitis. The concentrations of LBP in both biological fluids were increased within 12 h of inoculation of udder quarters with LPS from *E. coli (Bannerman et al., 2003)* and inoculation with another Gram$^-$ bacterium, *P. aeruginosa* caused elevation in LBP in milk and serum.

Monitoring the changes in serum LBP and SAA concentrations has also been used to characterize the innate immune response to experimental induction of mastitis with *Mycoplasma bovis* infection *(Kauf et al., 2007)*. The increases in serum LBP and SAA were compared along with other markers of the innate immune response, such as cytokines, between Holstein and Jersey breeds of dairy cows. The innate responses to inoculation of udders with *E. coli* were similar in both breeds *(Bannerman et al., 2008)*. Lipopolysaccharide binding protein has also been shown to be raised in dairy cows with natural clinical and subclinical mastitis and within the latter group the LBP levels were associated with SCC *(Zeng et al., 2009)*.

1.8.1 Biological Functions

The main role of LBP is to modulate the innate immune response. The structure of LBP includes an LPS binding domain involved in binding and transfer of LPS to the surface of monocytes/macrophages and granulocytes *(Lamping et al., 1996)*. LPS is an amphiphilic membrane phospholipid that forms aggregates in aqueous environments such as tissue culture medium or blood. Spontaneous diffusion of LPS monomers from these aggregates to CD14 occurs at a very slow rate *(Hailman et al., 1994)*. The plasma protein LBP, however, dramatically accelerates binding of LPS monomers from aggregates to CD14. It is believed to act by facilitating LPS monomerization and subsequent presentation to CD14 of macrophages (Fig. 1.13) and PMN *(Hailman et al., 1994 and 1996; Weiss, 2003)*, which, together with MD-2, function as relaying and sensing receptors for LPS in a pathway that ultimately leads to activation of TLR-4 and the initiation of an inflammatory response by the host *(Zweigner et al., 2006)*. LBP

transfers LPS to soluble CD14, resulting in activation of membrane CD14-negative cells such as endothelial and epithelial cells *(Pugin et al., 1993)*. It has been reported that a single LBP molecule is able to transport hundreds of LPS molecules to CD14, and that LBP is not consumed by this reaction *(Tobias et al., 1995)*. LBP also accelerates the transport of LPS into high-density lipoprotein, leading to neutralization of LPS *(Wurfel et al., 1994)*. This function of LBP may be protective during severe sepsis, as suggested by animal studies *(Jack et al., 1997; Lamping et al., 1998)*. LBP has also been demonstrated to facilitate LPS transfer to chylomicrons, an event that leads to both detoxification and enhanced removal of circulating LPS *(Harris et al., 1990, 1993; Vreugdenhil et al., 2003)*.

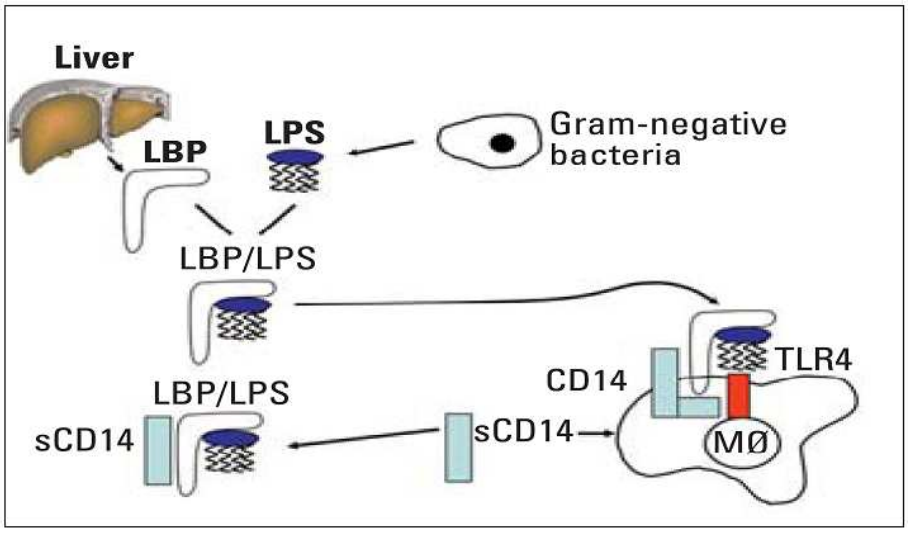

Figure 1.13: LPS recognition system. Abbreviation: LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; CD, cluster of differentiation; sCD14, soluble CD14; TLR4, toll-like receptor 4; MØ, macrophage *(Zeldin et al., 2006)*.

The binding and transfer of bacterial components by LBP is not limited to LPS-carrying bacteria but also includes Gram-positive microorganisms such as *S. pneumoniae* and

spirochetes like *B. burgdorferi*, clearly expanding its role to an important soluble pattern-recognition molecule *(Zweigner et al., 2006)*. In addition to LPS, LBP can also bind viable bacteria. Thus, LBP-binding to *Salmonella spp*. and *Klebsiella pneumoniae* has been demonstrated, which results in phagocytosis and clearance of these microorganisms *(Fan et al., 2002; Wright et al., 1989)*. In a respiratory tract infection model it could be demonstrated that the binding of LBP to one colony of nontypeable *Haemophilus influenzae* already induced strong signaling in the host cells *(Lazou et al., 2001)*.

The immunomodulatory activity of LBP is probably far more complex, and still not completely understood. LPB switches from a pro-inflammatory to an anti-inflammatory role depending on its concentration. Low concentrations of LBP fulfill a pro-inflammatory role. On the contrary, high concentrations of LBP, like those that can be found during systemic reaction have an opposite, inhibitory role *(Lamping et al., 1998)*. The up-regulation of LBP should then be regarded as one of the defense strategies of the host aiming to control a disproportionate inflammatory response.

There are few studies have been published evaluating the value of LBP as a diagnostic marker in patients with systemic inflammatory response syndrome (SIRS) of non infectious versus infectious origin and as potential prognostic marker predicting outcome. Investigation in pediatric patients and neonates suggest that LBP is a specific and sensitive marker that helps to differentiate between SIRS and bacterial infection *(Pavcnik-Arnol et al., 2004*). Especially in preterm neonates and in pediatric oncologic patients at the onset of febrile neutropenia, increased serum LBP concentrations may serve as an early diagnostic marker of bacterial infections *(Behrendt et al., 2004; Oude Nijhuis et al., 2003)*. In adult patients admitted on intensive care units (ICUs) increased

serum LBP concentrations correlated with the onset of bacteremia or severe sepsis and septic shock *(Froon et al., 1995)*. In a forensic study LBP has been shown to be a valuable marker for the post mortem diagnosis of sepsis *(Reichelt et al., 2005)*.

As other APPs, LBP may also act as an opsonin by binding to *Salmonella spp* and *K. pneumoniae* and promoting their phagocytosis *(Wright et al., 1989; Fan et al., 2002)*.

1.9 OTHER ACUTE PHASE PROTEINS

1.9.1 C-Reactive Protein

C-reactive protein (CRP), named for its capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumoniae*, was the first acute phase protein to be described and is an exquisitely sensitive systemic marker of inflammation and tissue damage *(Pepys and Baltz, 1983)*. CRP is synthesized by hepatocytes. There is also evidence that this protein can be extrahepatically synthesized by human macrophages, lymphocytes and monocytes *(Egenhofer et al., 1993; Kuta and Baum, 1986; Kolb-Bachofen et al., 1995)*. It is a pentameric protein consisting of five non-covalently bonded identical subunits with an overall molecular weight of approximately 11.8 kDa *(Kolb-Bachofen, 1991)*. It binds phosphocholine and therefore can recognize some foreign bacteria as pathogens, but also the phospholipid constituents of damaged cell *(Thompson et al., 1999)*. The protein has other sites that activate the classical complement pathway, interact with specific receptors on phagocytic cells to mediate phagocytosis, or induce the production of anti-inflammatory cytokines, thereby linking non-specific innate immunity

with specific adaptive immunity *(Du Clos and Mold, 2001)*. CRP inhibits chemotaxis and the respiratory burst of neutrophils *(Mortensen and Zhong, 2000)*.

1.9.2 Ceruloplasmin

Ceruloplasmin (Cp) which is 132 kDa monomer, an abundant plasma glycoprotein and contains about seven copper atoms per molecule, and in healthy adults accounts for up to 95 % of the total circulating copper *(Church et al., 1984)*. Like other acute phase proteins in addition to liver it is also synthesized from several extra-hepatic tissues like mammary gland *(Jaeger et al., 1991)*, uterus *(Schilsky et al., 1992)*, placenta *(Aldred et al., 1987)*, testes *(Fleming and Gitlin, 1990)* and monocytic cells *(Mazumder et al., 1997)*. Actually Cp is a copper containing ferroxidase that oxidizes toxic ferrous iron to its non-toxic ferric form *(Patel et al., 2002)*. It protects tissues from iron-mediated free radical injury and is involved in various antioxidant and cytoprotective activities *(Inoue et al., 1999)*. Cp may act as an anti-inflammatory agent by reducing the number of neutrophils attaching to the endothelium and by acting as an extracellular scavenger of peroxide *(Broadley and Hoover, 1989; Segelmark et al., 1997)*.

1.9.3 Fibrinogen

Fibrinogen (Fb) is synthesized in the high molecular weight form (HMW-fibrinogen, MW 340 kDa, ± 70 % of total fibrinogen). Partial degradation of the carboxy terminus of the fibrinogen Aa-chain (aC) results in two forms of fibrinogen in the circulation with decreased molecular weights: degradation of one of the Aa-chains gives the low

molecular weight form (LMW-fibrinogen, MW 305 kDa, ± 26 % of total fibrinogen) and degradation of both Aa-chains results in the LMW form (MW 270 kDa, ± 4 % of total fibrinogen) *(Nieuwenhuizen, 1995)*. The soluble protein Fb is an acute phase protein synthesized in liver and circulates in the blood to provide the material from which the insoluble fibrin clot is formed during blood coagulation. Fb is involved in homeostasis, providing a substrate for fibrin formation, and in tissue repair, providing a matrix for the migration of inflammatory related cells *(Thomas, 2000)*. Fb specifically binds to CD11/CD18 integrins on the cell surface of migrated phagocytes, thereby triggering a cascade of intracellular signals that lead to enhancement of degranulation, phagocytosis, antibody dependent cellular cytotoxicity and delay of apoptosis *(Sitrin et al., 1998; Rubel et al., 2001)*.

1.10 NEGATIVE ACUTE PHASE PROTEINS

1.10.1 Transferrin

Transferrin (Tf) is a glycoprotein of 80 kDa. The liver is the main source of manufacturing Tf, but other sources such as the brain also produce this molecule. The main role of Tf is to deliver iron from absorption centers in the duodenum and red blood cell macrophages to all tissues. Predominantly, transferrin plays a key role where erythropoiesis and active cell division occur. In order for iron ion to be introduced into the cell a carrier protein is used, known as a Tf receptor. The receptor helps maintain iron homeostasis in the cells by controlling iron concentrations *(Macedo and de Sousa,*

2008). Tf is found in the mucosa and binds iron, thus creating an environment low in free iron, where few bacteria are able to survive. The levels of Tf decreases in inflammation *(Ritchie et al., 1999)*, seeming contradictory to its function. Whereas Tf is usually considered to be a negative App, there is evidence that chicken Tf (ovo-Tf) increases in response to inflammation *(Tohjo et al., 1995; Xie et al., 2002)*.

1.10.2 Albumin

Bovine serum albumin (BSA), a large globular protein (66 kDa), consists of a single chain of 583 amino acids residues, and forms sub-domains by paired 17 disulfide bonds *(Peters, 1985)*. Albumin is synthesized largely in the liver, although no hepatic expression has been documented in several other tissues including mouse retina *(Dodson et al., 2001)*, mouse skeletal muscle *(Wagatsuma et al., 2002)*, human ovarian epithelial cells *(Varricchio and Stromberg, 1994)*, and bovine tracheal gland serous cells *(Jacquot et al., 1988)*, and in both healthy and mastitic mammary tissues *(Shamay et al., 2005)*.

While serum albumin concentration decreases during APR, it's concept as negative APP is debatable since the local expression of albumin is very often increased during inflammation for example, albumin concentration in milk climbs up during clinical and subclinical mastitis in cows *(Bannerman et al., 2003)*, sheeps *(Leitner et al., 2004)*, and goats *(Leitner et al., 2004a)*. Albumin concentration in milk also increases during functional transitions from lactation to involution and from involution to lactogenesis *(Sordillo et al., 1987)* and during inflammation *(Riollet et al., 2000; Watanabe et al., 2000)*. During these periods the mammary gland is exposed to high concentrations of free

radicals, and albumin may augment the antioxidant defenses of the gland *(Bounous, 2000)*.

The most well-known type of albumin is serum albumin. It is usually found in the blood or serum but it can also appear in other fluid compartments. Serum albumin is the most abundant blood plasma protein and is exclusively produced in the liver and forms a large proportion of all plasma protein *(Rothschild et al., 1988)*. The colloid pressure of plasma is maintained principally by the levels of circulating albumin *(West, 1991)*. Albumin also performs important metabolic functions in the transport of free fatty acids, bilirubin, and many drugs *(Chojkier, 2005; West, 1991)*. In a normal individual, approximately 15 g of albumin are synthesized daily by hepatocytes to maintain the albumin plasma steady state concentration (~4 mg/100 mL) *(West, 1991)*. BSA binds free fatty acids, other lipids and flavor compounds that can alter heat denaturation of the protein *(Kinsella, 1989)*. Also, BSA may play a role in lipid oxidation *(Smith et al., 1992)* and in the maintenance of blood pH *(Figge et al., 1991)*.

1.11 MiRNAs: EMERGING POTENTIAL BIOMARKERS

MiRNAs are single-stranded, non-coding RNAs of about 22 nt in length primarily functioning as regulators of gene expression *(Luo et al., 2010)*. The canonical biogenesis of miRNA employs stepwise processing from the primary hairpin-shaped transcripts (pri-miRNA) by RNAse III-type Drosha to shorter pre-miRNA. These are transferred from the nucleus to the cytoplasm via exportin 5 and cleaved by Dicer, another RNAse III member *(Bartel, 2004)*. The resulting RNA duplex is loaded into an Argonaute (Ago) protein in a three-step process of RNA-induced silencing complex (RISC) assembly *(Kim et al., 2009; Kwak and Tomari, 2012)*. Based on the thermodynamic stability of base pairs at the 5' end, the guide strand remains as a mature miRNA within the functional RISC complex while the passenger strand is mostly discarded *(Kim et al., 2009*; *Kawamata and Tomari, 2010)*.

The seed region of the mature miRNA interacts with a complementary sequence in the 3' un-translated region (3' UTR) of the messenger RNA (mRNA). Exceptionally, miRNAs exert their function when the binding sites are located in 5' UTR or within coding regions *(Filipowicz et al., 2008; Kloosterman et al., 2004)*. Perfect complementarity specifies further mRNA cleavage that dominates in plants. In animals, the consequences of partial seed match are less clear *(Bartel, 2004; Filipowicz et al., 2008)*. Initially, miRNAs were thought to repress translation with little or no influence on mRNA levels *(Pillai et al., 2007)*. It is becoming clear that the effects of miRNAs on protein synthesis can result also from mRNA de-adenylation which promotes de-capping, destabilization, and mRNA degradation *(Wu et al., 2006; Behm-Ansmant et al., 2006)*. Latest data have shown that only a small fraction of repression (11-16 %) was attributable to reduced

translational efficiency *(Guo et al., 2010)*. Instead, lowered mRNA levels account for most (≥84 %) of the decreased protein production *(Guo et al., 2010)*. Therefore, destabilization of target mRNA appears to be the predominant reason for reduced protein output *(Guo et al., 2010)*. In contrast to the canonical role of miRNAs in repression, miRNAs can also enhance translation under specific conditions *(Vasudevan et al., 2007)*.

Besides the above mentioned pathway, several alternative miRNA biogenesis pathways have been discovered *(Kim et al., 2009)*. Mirtrons, small RNAs embedded in short introns, are cleaved by splicing and other ribonucleolytic machinery (instead of Drosha processing) to yield Dicer substrates for further miRNA biogenesis *(Westholm and Lai, 2011)*. In addition, a Dicer-independent miRNA biogenesis that requires Ago2 catalytic activity has been described *(Cifuentes et al., 2010; Cheloufi et al., 2010)*.

Initially, miRNA* passenger strands were only considered as byproducts in the miRNA biogenesis executed for degradation *(Okamura et al., 2008)*. Because strand selection is often not a strict process, some hairpins can produce miRNAs from both strands at comparable frequencies *(Kim et al., 2009)*. The final fate of miRNA* strand, either degraded as a 'carrier' strand or profiled as a functional miRNA, may be destined across evolution *(Guo and Lu, 2010a)*. miRNA*, although less abundant than guide strands, are present at physiologically relevant levels and can associate with Ago proteins. Importantly, they are of the inhibitory activity in both *in vitro* and *in vivo* models with impact on regulatory networks *(Okamura et al., 2008; Yang et al., 2011)*.

As fine tuning regulators of gene expression, miRNAs are involved in crucial cellular processes and their dysregulation has been described in many diseases. The aberrant

miRNA expression can be affected by chromosomal abnormalities, mutations, single nucleotide polymorphisms (SNPs), transcriptional deregulation, defects in the miRNA biogenesis machinery and epigenetic changes *(Iorio et al., 2010)*. The accumulating evidence showed the employment of miRNA in the pathophysiology of inflammatory rheumatic diseases and other auto-inflammatory conditions *(Tili et al., 2008; Baxter et al., 2012)*.

The expression of miRNAs in inflammatory and immune responses can be used as biomarkers to identify, prematurely, pathology's state.

Filková and colleagues *(Filková et al., 2012)* showed that, as shown by an increasing number of *in vitro* and *in vivo* studies, miRNA's alteration in immune as well as in resident cells at the site of inflammation, contributes to the patho-physiology of rheumatoid arthritis.

Incorporated in microvesicles, in complexes with Ago2 or HDL, miRNAs show high stability in body fluids. MiRNAs possess many features of ideal biomarkers that have already been appreciated in several studies *(Filková et al., 2012)*.

Mature miRNAs play key roles in various cellular processes commonly implicated in cancer, such as differentiation, cell growth, angiogenesis, epithelial-to-mesenchymal transition (EMT) and invasion *(Valladares-Ayerbes et al., 2012)*. A large amount of data has revealed the correlation between specific tumors and differential miRNA expression profiles, thus providing a new class of disease-specific biomarkers *(Lu et al., 2005; Rosenfeld et al., 2008)*. An increasing number of studies analyzing the miRNA expression profiles in gastrointestinal tumors, including Gastric Cancer, and their potential clinical relevance have been reported *(Li et al., 2010; Ueda et al., 2010)*.

Accumulating reports have indicated that miRNAs are detectable in blood and that circulating miRNAs have the potential to be new biomarkers in patients with different diseases including cancer. Circulating miRNAs must demonstrate different hallmark characteristics to considered reliable biomarkers *(Gilad et al., 2008;* Etheridge *et al., 2011)*: (1) stable and readily quantifiable in clinical samples; (2) expressed by cancer cells at moderate or high levels; (3) present at undetectable or very low levels in specimens from individuals without cancer; (4) provide a predictive or prognostic clinical information; and (5) exhibit biological functions mechanistically linked to tumor progression.

miR-200b belongs to the miR-200 family member which controls epithelial-mesenchymal transition *(Brabletz and Brabletz, 2010)*. Loss of miR-200b induces a mesenchymal phenotype, enhanced motility, and invasiveness of wide variety of transformed cells *(Brabletz and Brabletz, 2010)*. Chan's group *(Chan et al., 2012)* and others have reported that miR-200b not only modulates cell motility but also controls several pro-angiogenic proteins regulating endothelial cell function.

Several studies have reported the involvement of miR dysregulation in the diabetes mellitus associated pathogenic angiogenesis. In type 2 diabetic Goto-Kakizaki rats, up-regulation of miR-320 inhibited angiogenic response of myocardial microvascular endothelial cells by targeting insulin-like growth factor 1 *(Wang et al., 2009)*. Elevation of miR-503 silenced cyclin E1 and cdc25A, subsequently inhibiting angiogenesis in diabetic ischemic muscle *(Caporali et al., 2011)*. High glucose induced loss of miR-93, a miR-targeting renal endothelial VEGF, resulted in aberrant angiogenesis *(Long et al., 2010)*. It was demonstrated that diabetic wound-edge endothelial cells suffer from

silencing of GATA2 and VEGFR2, which is consistent with previous findings, *(Keswani et al., 2004; Krishnan et al., 2007)* via a miR-200b-dependent mechanism.

2. PhD thesis rationale

The aim of this doctoral research was identify biomarkers for evaluating the health of farm ruminants and quality of products. One of the most important factors, which determine the quality of the milk, is represented by the sanitary conditions of lactiferous animals; in this context, a rapid identification and quantification of acute phase proteins can provide useful information on the health status of animal.

It should also not be underestimated that milk from non bovine species, such as goat's milk for example, is used for the production of typical products without it being subjected to a preliminary thermal treatment.

From a clinical perspective, the availability of a quick, and reliable, diagnostic marker specific for inflammatory diseases is of paramount importance. Acute phase proteins (APP) are good candidates. Regrettably, little information is available about their localization or local expression. This information is precious, given the very different behavior of acute phase proteins among different species. APPs can be employed in the assessment of animal health and welfare and ante-mortem as an aid to meat inspection. A critical mass of knowledge in the use of APPs as biomarkers of inflammatory conditions of domestic animals has accumulated over recent years. While information about APP location and production is still lacking, there is now sufficient understanding of the pathophysiology of the inflammatory response to support the use of these compounds as diagnostic tools in clinical settings. With the insights provided by ongoing

research in the area, it is likely that these analytes will be increasingly used in the diagnosis and prognosis of both companion and farm animal disease.

On this background, the present investigation can be divided into two principal studies:

1. Localization of APPs.

In this study the localization of APP in extra hepatic tissues was assessed. The experiments were organized as follows:

- Distribution of acute phase proteins in the bovine forestomachs and abomasums;

- Expression of SAA and Hp RNA in bovine tissues (and evaluation of suitable reference genes).

Given the greater availability of structural information, such as sequence knowledge and other preliminary data, as well as biological specimens, this part of the thesis was carried out on bovine species.

2. Individuation of specific molecules that can be used as biomarkers.

This second part was more focused on the search for specific inflammatory biomarkers. This part of the thesis was sub-divided as follows:

- *Escherichia coli* lipopolysaccharides and *Staphylococcus aureus* enterotoxin B relationship with inflammatory microRNAs in bovine monocytes;

- Development of a highly sensitive sandwich ELISA to quantify the goat and bovine AGP and SAA in serum.

3. Distribution of acute phase proteins in the bovine forestomachs and abomasum

3.1 OBJECTIVES AND EXPERIMENTAL DESIGN

The aim of this study was to investigate and to perform more detailed examination of expression of the four major bovine APPs: haptoglobin (Hp), serumamyloid A (SAA), lipopolysaccharide-binding protein (LBP) and α-1 acid glycoprotein (AGP) in the normal bovine forestomachs and abomasum. The experimental steps were as follows:

1) Tissue collection and preservation;
2) Qualitative and quantitative mRNA expression;
3) Western blot analysis;
4) Immunohistochemistry.

3.2 INTRODUCTION

The gastrointestinal tract fulfils divergent roles of nutrient absorption and host defence. The mucosal surfaces of the forestomachs and intestines of ruminants are in continuous contact with a vast, diverse and dynamic microbial community. Since microbes can cross the epithelial surface through the intestinal barrier, protective immune responses must be raised against pathogens, whereas there is a need to tolerate innocuous antigens from commensal bacteria. Intestinal defences include epithelial derived antimicrobial

peptides, cytokines and acute phase proteins (APPs) *(Dommett et al., 2005)*. APPs belong to a large family of structurally unrelated proteins that are produced as part of the systemic inflammatory response and play a role in modulating innate immunity and scavenging inflammatory by-products *(Gabay and Kushner, 1999)*. They are produced mainly by the liver after stimulation by pro-inflammatory cytokines *(Baumann and Gauldie, 1994)*, but extra-hepatic expression also has been reported *(McDonald et al., 2001; Upragarin et al., 2005; Lecchi et al., 2009; Rahman et al., 2010)*. There is little information on the involvement of the ruminant gastric mucosa in innate immunity. The mucosa of the three forestomachs (rumen, reticulum and omasum) is lined by stratified, squamous, keratinised epithelium and the mucosa of the abomasum is lined by simple, glandular, columnar epithelium *(Scala et al., 2011)*. The rumen contains >1010 commensal microbiota/g contents and the mucosal surface represents an important line of defence against the penetration of microorganisms *(Krause and Russell, 1996)*. There is no organised lymphoid tissue in the mucosa of the forestomachs and abomasum. Scattered lymphocytes and Langerhans' cells are present in the forestomachs *(Josefsen and Landsverk, 1996)* and there are scattered mast cells and lymphocytes in the abomasum *(Balic et al., 2000)*. The four major bovine APPs are haptoglobin (Hp), serum amyloid A (SAA), lipopolysaccharide-binding protein (LBP) and α-1 acid glycoprotein (AGP). Previous studies have identified expression of LBP *(Rahman et al., 2010)* and AGP *(Lecchi et al., 2009)* in the stomachs and intestinal tract of ruminants. In the present study, we performed a more detailed examination of expression of Hp, SAA, LBP and AGP in the normal bovine forestomachs and abomasum.

3.3 MATERIALS AND METHODS

Tissue collection and preservation

Bovine gastric tissues were collected from four clinically healthy animals at a local abattoir. The tunica mucosa was mechanically separated from the sub-mucosa with a scalpel and portions of tissue were preserved in RNAlater (Sigma-Aldrich) and stored at -80 °C. Samples for Western blot analysis were collected immediately into liquid nitrogen and stored at -80 °C. Samples for immunohistochemistry were embedded in Killik (Bio-Optica), frozen in liquid nitrogen-cooled isopentane (Sigma-Aldrich) and stored at -80 °C. Unless otherwise stated, all subsequent procedures were conducted at room temperature.

Qualitative and quantitative mRNA expression

Total RNA was extracted using TriZol (Invitrogen) and treated with DNase I (Invitrogen). Total RNA was quantified using a NanoDrop ND-1000 UV-vis spectrophotometer. Reverse transcription (RT) was carried out with 1 µg RNA using the iSCRIPT cDNA Synthesis Kit (BioRad). The cDNA was used as the template for PCRs, which were performed in 10 µl final volumes containing 1 µl buffer (Vivantis), 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 1 µM each primer and 0.025 U Taq polymerase (Vivantis). The same primers were used in qualitative and quantitative PCR for SAA and LBP, whereas different primers were used for Hp and AGP (Table 1). PCR conditions were 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s (Eppendorf Mastercycler). PCR products were visualised on 1.9 % agarose gels stained with ethidium bromide. Quantitative reactions were performed using 20 µl Eva

Green mix (BioRad) and 450 nM each primer (SAA, LBP, Hp_RT, AGP_RT and ß-actin; Table 3.1). Each sample was tested in duplicate. To evaluate PCR efficiency, fourfold serial dilutions were prepared from reference samples. The thermal profiles for each target gene were 95 °C for 90 s, 50 cycles of 95 °C for 5 s and 60 °C for 10 s; conditions for melting curve construction were 55 °C for 60 s then 80 cycles starting at 55 °C and increasing 0.5 °C each 10 s. Results were compared using the Δ-Δ Cq method *(Giulietti et al., 2001)*.

Name	Fragment size (base pairs)	Sense	Antisense
SAA	121	5′-CCTGCTGGCCTGCCTGAC-3′	5′-GCTGCCTTCTGAGGACAGAG-3′
LBP	130	5′-CCTGATTCTAGCATTCGACAG-3′	5′-GGCTGAAGTTCAGGCACG-3′
Hp	337	5′-GCAGCTTTCCCTGGCAGG-3′	5′-CCAGACACATAACCCACACG-3′
Hp_RT	67	5′-CCAAGATGGTCTCCCAGCAT-3′	5′-GTGAGGAGCCATCGTTCAATTG-3′
AGP	226	5′-GCATAGGCATCCAGGAATCA-3′	5′-GCACCGAAACAAACTTTATTGATGC-3′
AGP_RT	112	5′-GCATAGGCATCCAGGAATCA-3′	5′-TAGGACGCTTCTGTCTCC-3′
β-actin	133	5′-CCAAAGCCAACCGTGAGA-3′	5′-CCAGAGTCCATGACAATGC-3′

Table 3.1: Sequences of oligonucleotide primers for acute phase proteins.

SAA, serum amyloid A; LBP, lipopolysaccharide-binding protein; Hp, haptoglobin; AGP, a-1 acid glycoprotein. SAA, LBP, Hp, AGP and ß-actin primers were designed on the basis of GenBank sequences (Accession Numbers NM181016.3, NM001038674.1, NM_001040470.1, AM403243 and BC142413.1, respectively). Hp_RT primers sequences are from Eckersall *(Eckersall et al., 2006)*.

Western blot analysis

Antibodies validated in cattle for detection of APPs by Western blot analysis and immunohistochemistry are listed in Table 2. Samples for Western blot analysis were prepared from aliquots of 50-100 mg tissues using protease inhibitors (Sigma-Aldrich), as previously described *(Rahman et al., 2008)*. The protein content of the supernatant was quantified at A 280 nm. Aliquots of 25-50 µg/ml were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotted

onto nitrocellulose membranes. The membranes were immunolabelled for the presence of APPs using specific antibodies (Table 3.2) and immunoreactive bands were visualised by enhanced chemiluminescence (ECL) using Immobilon Western Chemiluminescence substrate (Millipore). To confirm that an equal amount of protein was loaded in each lane, membranes were stripped and immunolabelled with mouse anti ß-actin antibody (1:10.000). Liver lysates were used as positive controls.

Name	Western blot analysis			Immunohistochemistry	
	Primary antibody	Incubation time	Reference	Primary antibody	Incubation time
Mouse anti-bovine SAA (C100-8)	1:100 (3.9 g/ml)	60 min	McDonald et al. (1991)	_	_
Mouse anti-human LBP (biG42)	1:200 (5 µg/ml)	45 min	Rahman et al. (2010)	_	_
Rabbit anti-bovine Hp	1:2,000 (1.37µg/ml)	45 min	_	1:200 (13.75µg/ml)	Overnight
Rabbit anti-bovine AGP	1:2,000 (1.65µg/ml)	45 min	Ceciliani et al. (2007a)	1:200 (16.5µg/ml)	Overnight
Mouse anti-b-actin (CP01)	1:10.000	45 min	Lecchi et al. (2008)	_	_

Table 3.2: Primary antibodies for Western blotting and immunohistochemistry.

SAA, serum amyloid A; LBP, lipopolysaccharide-binding protein; Hp, haptoglobin; AGP, α-1 acid glycoprotein.

Immunohistochemistry

Immunohistochemistry for AGP and Hp was performed on cryostat sections incubated in 0.3% H_2O_2 in methanol (Sigma-Aldrich) for 30 min to block endogenous peroxidase activity. Non-specific binding sites were blocked with 10 % normal goat serum (Sigma-Aldrich) for 30 min and sections were incubated overnight at 4 °C in the presence of specific antibodies (Table 2). Staining of specific proteins was carried out with diaminobenzidine (DAB, Vector Laboratories) after incubation with peroxidase-conjugated secondary antibodies (Sigma-Aldrich). Nuclei were counterstained with Mayer's haematoxylin (DDK Italia). Sections were mounted in Poly-mount (Polysciences) and examined using a Nikon Eclipse E600 microscope. Bovine liver was used as a positive control. Primary antibodies were omitted for negative controls.

3.4 RESULTS

Expression of acute phase protein mRNA in the bovine forestomachs and abomasums

Expression of Hp, SAA, LBP and AGP RNA was detected by qualitative RT-PCR in the mucosa of the bovine forestomachs and abomasum (Fig. 3.1). Quantitative RT-PCR was used to determine the relative expression of Hp, SAA, LBP and AGP RNA after normalisation against ß-actin (Fig. 1). Increased amounts of SAA and LBP RNA relative to the liver were expressed in the mucosa of the forestomachs and abomasum; the highest levels of SAA were detected in the omasum and abomasum, whereas the highest levels of LBP were detected in the omasum. Expression of AGP and Hp in the mucosa of the forestomachs and abomasums was negligible compared to the liver.

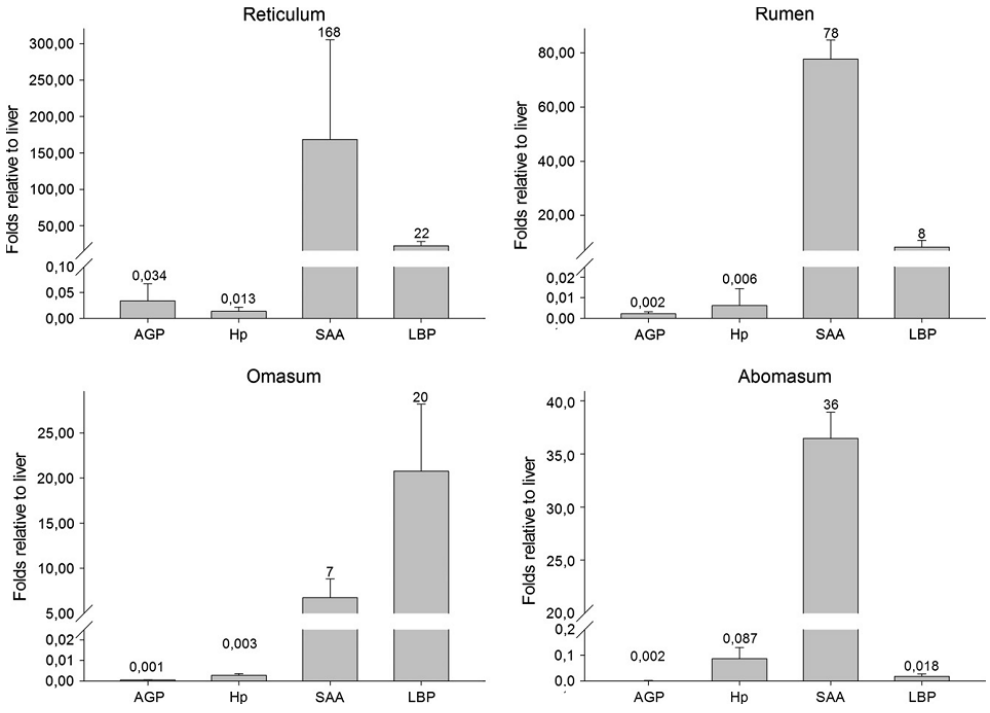

Fig. 3.1: Relative expression of serum amyloid A (SAA), lipopolysaccharide-binding protein (LBP), haptoglobin (Hp) and α-1 acid glycoprotein (AGP) in the bovine forestomachs mucosa by real-time PCR. Values are expressed as fold change relative to liver and normalised using ß-actin as the reference gene. Data are means ± standard error of four experiments.

Expression of acute phase proteins in the bovine forestomachs and abomasums

Hp, LBP and AGP were detected by Western blot analysis in all samples of mucosa from the forestomachs and abomasum (Fig. 3.2). AGP was detected as a low molecular weight (MW) immunoreactive band of 21 kDa expressed mainly in the rumen, reticulum and omasum, which corresponded to the expected MW of bovine de-glycosylated AGP (21.3 kDa). In addition, there was a higher MW band of 43 kDa expressed mainly in the omasum and abomasum, which corresponded to fully glycosylated AGP. Hp was detected as two major bands of 16 and 45 kDa, corresponding to the a and b chains of human Hp *(Levy et al., 2010)*, respectively, in the rumen and abomasum. Several high MW bands

were also detected in the forestomachs and abomasum; these were the predominant immunoreactive bands in the reticulum and omasum and are likely to be due to association of the 16 and 45 kDa sub-units with albumin *(Eckersall and Conner, 1990).* Immunoreactivity for LBP was evident in the forestomachs and abomasum at 55-60 kDa (corresponding to the expected MW of LBP), with the highest band intensity in the abomasum. LBP immunoreactivity was also evident at 18 kDa (low MW bands) and 70-80 kDa (high MW bands). SAA was detected only in the abomasum, with a MW of 14 kDa (Fig. 3.2). AGP and Hp were localised by immunohistochemistry in the superficial layers of the stratified squamous epithelium of the forestomachs and in the glandular epithelium of the abomasum (Fig. 3.3).

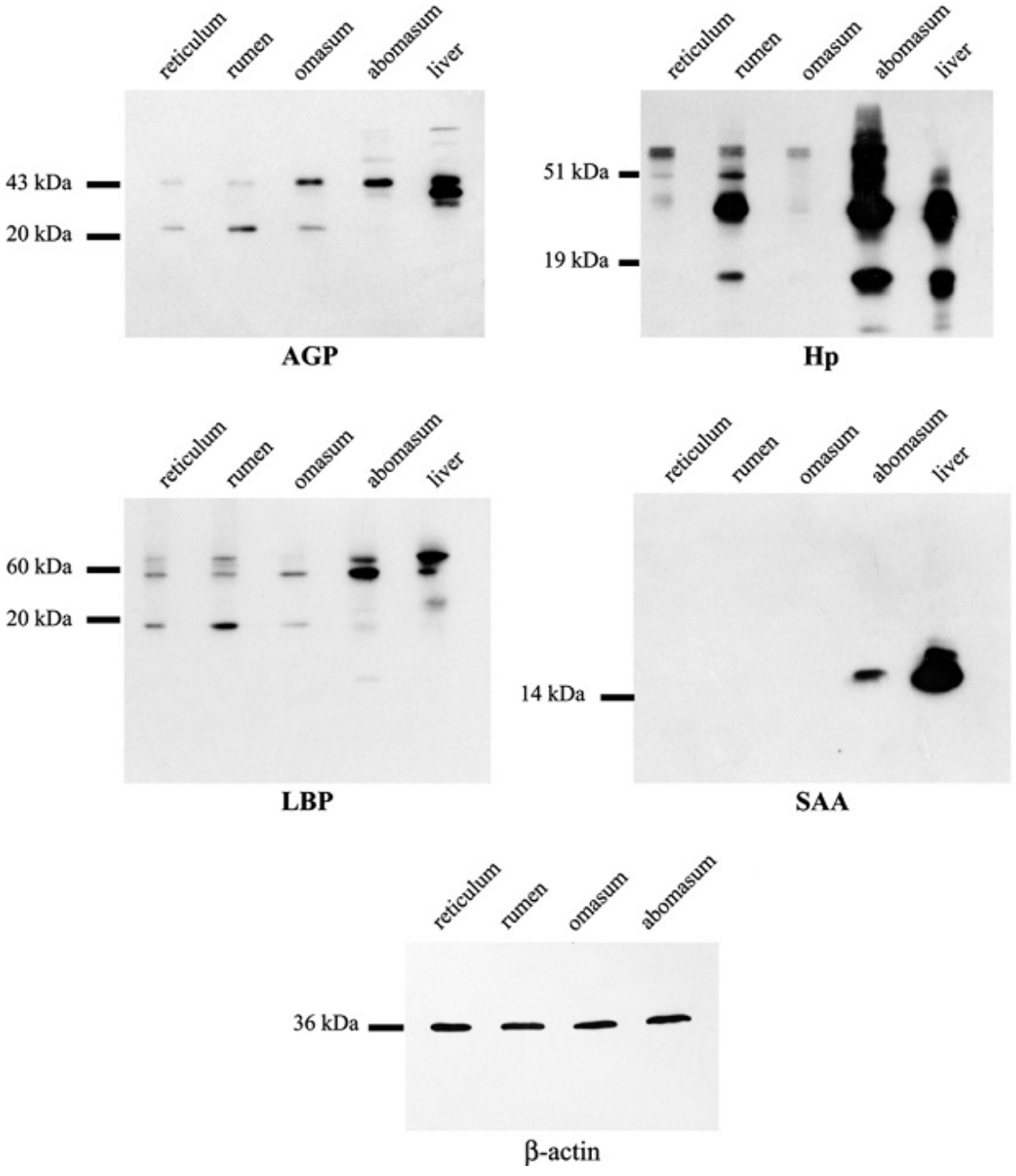

Fig. 3.2: SDS-PAGE and Western blot analysis of the forestomachs and abomasum mucosal lysates. Acute phase proteins were identified after immunostaining using antibodies listed in Table 1 and detection by electrochemiluminescence.

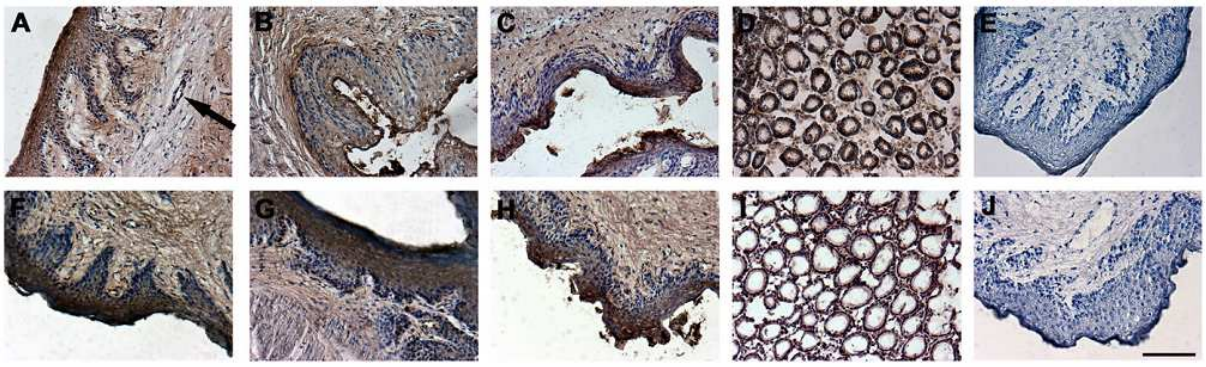

Fig. 3.3: Immunolocalisation of α-1 acid glycoprotein (AGP) and haptoglobin (Hp) in the bovine forestomachs and abomasum. Representative images demonstrate AGP (A-E) and Hp (F-J) localisation in the bovine rumen (A and F), reticulum (B and G), omasum (C and H) and abomasum (D and I). Note the staining of endothelial cells of blood vessels (arrow). E and J show negative control staining in the rumen (E) and omasum (J). Bar = 100 μm.

3.5 DISCUSSION

APPs exhibit binding and immunomodulatory properties and are involved in the first stages of the innate immune response *(Murata et al., 2004; Petersen et al., 2004)*. Thus, APPs expressed in the gastric mucosa might contribute to homeostasis of the host-microbial interface. In this study, APPs were detectable in the mucosa of the bovine forestomachs and abomasum, suggesting that these tissues are capable of generating a local acute phase response. In view of its large surface area, the mucosa of the ruminant forestomachs and abomasum may be an important source of APPs during diseases such as sub-acute ruminal acidosis *(Gozho et al., 2005)* and abomasal parasitism *(Conner et al., 1989)*. Hp and SAA are the major APPs released during inflammation in cattle *(Petersen et al., 2004)*. SAA is a multifunctional protein involved in several immunomodulatory functions, including opsonisation of bacteria *(Hari-Dass et al., 2005;*

Shah et al., 2006; Molenaar et al., 2009). In the present study, SAA mRNA was demonstrated in the forestomachs and abomasum by quantitative PCR, but SAA protein could be identified only in the abomasum. Since SAA is expressed in a spatially and temporally restricted manner in tissues *(McDonald et al., 2001; Molenaar et al., 2009)*, it is possible that SAA mRNA accumulates constitutively in gastric epithelial cells, but is translated into active proteins only during pathological states.

The main activity of Hp is to bind haemoglobin, thus preventing renal damage and iron loss *(Levy et al., 2010)*, as well as inhibiting the utilisation of iron by pathogenic bacteria *(Eaton et al., 1982).* Expression of Hp protein was high in the forestomachs and abomasum, even though expression of Hp mRNA was negligible. It is possible that Hp may not be produced locally in the forestomachs or abomasum, but instead may be produced elsewhere, probably in the liver, then transported to the gastric mucosa. LBP is secreted by enterocytes and activates host defences against bacteria, as well as being involved in endotoxin tolerance *(Vreugdenhil et al., 1999; Zweigner et al., 2006).* The main site of expression of LBP mRNA in the present study was the omasum, confirming previous findings *(Rahman et al., 2010)* (Fig. 2), whereas the highest protein expression was evident in the abomasum.

AGP is a binding protein with immunomodulatory functions expressed at high levels in the liver and moderate levels in the mammary gland, salivary glands, pancreas, spleen, lungs and uterus of cattle *(Ceciliani et al., 2007b; Lecchi et al., 2009).* The present study also shows that AGP is expressed in the bovine forestomachs. Western blot analysis demonstrated several isoforms of APPs that differed from those expressed in the liver. It is possible that glycosylation patterns of APPs are specific for the site of origin *(Ceciliani et al., 2007b; Cooray et al., 2007; Rahman et al., 2010).*

3.6 CONCLUSIONS

The three bovine forestomachs and the abomasum produce APPs. Although expression of SAA mRNA was evident in the forestomachs and abomasum, SAA protein could be identified only in the abomasum. Expression of Hp protein was high in the forestomachs and abomasum, even though expression of Hp mRNA was negligible. The main site of expression of LBP mRNA was the omasum, whereas the highest protein expression was evident in the abomasum. AGP was expressed at low levels in the bovine forestomachs. Western blot analysis demonstrated several isoforms of APPs that differed from those expressed in the liver. On the basis of their lipid-binding and immunomodulatory activities, the expression of APPs in the bovine forestomachs and abomasums may contribute to regulation of the innate immune response against bacteria and parasites.

Moreover, the finding that forestomachs and abomasum mucosa may produce APP may support their use as possible biomarkers for ruminant gastric diseases.

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Distribution of acute phase proteins in the bovine forestomachs and abomasum

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ABSTRACT

Acute phase proteins (APPs) are produced mainly by the liver and their concentration is increased during the systemic inflammatory response. Expression of haptoglobin (Hp), serum amyloid A (SAA), lipopolysaccharide-binding protein (LBP) and α-1 acid glycoprotein (AGP) was determined in the mucosa of the normal bovine forestomachs and abomasum by qualitative and quantitative reverse transcriptase-PCR for mRNA and by Western blot analysis and immunohistochemistry for proteins. Although expression of SAA mRNA was evident in the forestomachs and abomasum, SAA protein was identified only in the abomasum. Expression of Hp protein was high in the forestomachs and abomasum, even though expression of Hp mRNA was negligible. The main site of expression of LBP mRNA was the omasum, whereas the highest protein expression was evident in the abomasum. AGP was expressed at low levels in the bovine forestomachs. Western blot analysis revealed a heterogeneous electrophoretic pattern for AGP, LBP and Hp, indicating that different stomach compartments produce isoforms that are different to those expressed by the liver. Expression of APPs by the bovine forestomachs and abomasum may contribute to regulation of the innate immune response against pathogens.

4. Widespread expression of SAA and Hp RNA in bovine tissues after evaluation of suitable reference genes

4.1 OBJECTIVES AND EXPERIMENTAL DESIGN

The aim of the present study was to assess the constitutive SAA and Hp mRNA expression by quantitative PCR (qPCR) in a wide panel of 33 bovine tissues, including gastrointestinal tract, respiratory system, urogenital system, mammary gland, hematopoietic system, central nervous system, eye, thyroid and heart.

Normalization of gene expression in different samples requires reference genes, which are stably expressed. Therefore, seven reference genes were investigated (ACTB, GAPDH, HMBS, SDHA, YWHAZ, SF3A1, EEF1A2) and three genes, namely SF3A1, HMBS and ACTB, were selected after assessing their stability with geNorm™ and NormFinder© softwares. The experimental steps were as follows:

1) Tissue collection and preservation;

2) Selection of reference gene;

3) Quantitative mRNA expression.

4.2 INTRODUCTION

The acute phase proteins (APPs) belong to a large family of structurally unrelated proteins, involved in the acute phase response (APR) to inflammation, a dynamic process induced by pro-inflammatory cytokines *(Gabay and Kushner, 1999)*. Serum amyloid A (SAA) and haptoglobin (Hp) are regarded as the two major APPs in most species, including cattle, their plasma concentrations rising up several folds in response to inflammation or infection *(Eckersal and Bell, 2010)*. SAA fulfills several immuno-modulatory functions *(Jensen and Whitehead, 1998)*, which include the opsonization of both $Gram^+$ and $Gram^-$ *(Hari-Dass et al., 2005; Molenaar et al., 2009)*, thus increasing the response of innate phagocytic cells to bacteria. Hp binds haemoglobin (Hb), and in as such inhibits the utilization of iron by pathogens *(Eaton et al., 1982)*.

While most of circulating SAA and Hp are likely to be produced by liver, extra-hepatic expression has been also reported *(Upragarin et al., 2005; Skovgaard et al., 2009)*. In cattle, local production has been identified in mammary gland *(Molenaar et al., 2009)*, adipose tissue *(Mukesh et al., 2010)*, reproductive system *(Lavery et al., 2004)*, forestomachs *(Dilda et al., 2012)*, leukocytes *(Cooray et al., 2007)* and in several other bovine tissues *(Berg et al., 2011)*.

The local expression of acute phase proteins has usually been linked to a pathological status. The main objective of this investigation was to study the mRNA expression of bovine SAA and Hp in non pathological tissues.

Accurate quantification of mRNA with quantitative PCR (qPCR) requires the identification of a proper panel of endogenous genes to be used as stable controls (reference genes, RGs). Set of bovine RGs are available, but they were mostly selected in

a given patho-physiological context *(Kadegowda et al., 2009)*. In other experiments the identification of RGs was tissue-specific *(Lisowski et al., 2008; De Ketelaere et al., 2006; Robinson et al., 2007; Spalenza et al., 2010; Pérez et al., 2008; Hosseini et al., 2010)*. The statistical softwares geNorm™ *(Vandesompele et al., 2002)* and NormFinder© *(Andersen et al., 2004)* has been developed to assess the appropriateness of RGs. GeNorm™ determines the expression stability of non-normalized control genes by assigning for each gene a gene stability measure (M). NormFinder© uses a model based approach to rank all RGs based on inter- and intra-group expression variations. Since the two softwares may deliver different results, and there is no universally accepted method to analyze the applicability of common stable RGs, a preliminary step of the present study was carried out to select a set of RGs that can be used for normalizing qPCR RNA expression data from different bovine tissues by comparing the output of two most widely utilized statistical softwares geNorm™ and NormFinder©.

4.3 MATERIALS AND METHODS

Bovine tissue samples, as listed in Table 4.1 were collected during routinely slaughtering procedures from two healthy Holstein Friesians dairy cows, seven year old, which were culled due low milk production. The healthy clinical status of the animals was assessed by ante-mortem inspection and the absence of gross findings recorded during common slaughterhouse procedures. Portions of each tissue were removed immediately after slaughtering, preserved in RNAlater® (Sigma-Aldrich Co.) and stored at -80 °C before RNA extraction. Bovine blood monocytes isolation was carried out as previously described *(Ceciliani et al., 2007a)*.

Gastrointestinal tract	Tounge	
	Salivary glands	Parotid glands Submandibular glands
	Esophagus	
	Intestine	Small intestine Ciecum - Colon
	Liver	
	Pancreas	
Respiratory system	Trachea	
	Lung	
Urogenital system	Kidney	Renal parenchyma Renal pelvis
	Bladder	
	Ovary	
	Uterus	
Integumentary system	Mammary gland	Cistern Teat Parenchyma Quarter Furstenberg's rosettes
	Skin	
Hematopoietic system	Bone marrow	
	Lymph nodes	
	Spleen	
	Monocytes	
Thyroid		
Cardiovascular system	Heart	
Central nervous system	Brain	
	Cerebellum	
Eye	Cornea	
	Tapetum lucidum	

Table 4.1.: Samples list.

Seven RGs were selected (ACTB, GAPDH, HMBS, SDHA, YWHAZ, SF3A1, EEF1A2) based on previous studies. The genes belong to different functional classes to reduce the chance that genes might be co-regulated (Table 4.2). The YWHAZ primers were defined by *Goossens et al. (2005)*, EEF2A1 by *Pérez et al. (2008)*, GAPDH by *Lecchi et al. (2008)* and ACTB by *Dilda et al. (2012)*. The HMBS, SDHA and SF3A1 were designed using primer 3 preventing possible secondary structures with the mfold Web Server (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) and ensuring the specificity of the sequence by BLAST.

SAA and Hp primers sequences were from *Dilda et al. (2012).*

Total RNA was extracted using Trizol® Reagent (Invitrogen) according to the manufacturer's protocol. Concentration of total RNA in each sample was quantified by NanoDrop ND-1000 UV-vis spectrophotometer (NanoDrop Technologies Inc.) and the purity was determined by evaluation of the ratio A260:A280. RNA quality was evaluated using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA); the RNA integrity number (RIN) was ≥ 7 for each sample. Genomic DNA was eliminated using deoxyribonuclease I (DNase I) (Invitrogen). The reverse transcription reaction was carried out on 1 µg RNA using iSCRIPT cDNA SYNTESIS Kit (BioRad). To perform gene testing, part of the reaction was diluted 1:2 and part was pooled. The pooled cDNA was obtained by mixing 2 µl of each sample listed in Table 1. The cDNA was used as template for PCR (Eppendorf Mastercycler) reactions as reported in Table 3. The same primers were used in qualitative and quantitative PCR (Table 4.2).

Symbol	Name	Accession number	Function	PCR efficency and regression coefficient (r^2)	Forward 5'→3'	Reverse 5'→3'
ACTB	Beta actin	BT030480.1	Cytoskeletal structural protein.	99.5% 0.994	CCAAAGCCAACCGTGAGA	CCAGAGTCCATGACAATGC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034	Oxidoreductase in glycolysis and gluconeogenesis.	102% 0.997	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAGT
HMBS	Hydroxymethyl-bilane synthase	NM_001046207.1	Heme syntesis, porphyrin metabolism.	100.8% 0.998	GAGAGGAATGAAGTGGACCTAG	GCATCATAGGGGCTCTCCC
SDHA	Succinate dehydrogenase complex, subunit A	NM_174178.2	Electron transporter in the TCA cycle and respiratory chain.	97.7% 0.996	TGTATAATAGCTCACGCCCG	TCTATCAGATGGCCTCCTCAG
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_174814	Signal transduction by binding to phosphorylated serine residues on a variety of signalling molecules.	92.3% 0.996	GCATCCCACAGACTATTTCC	GCAAAGACAATGACAGACCA
SF3A1	Splicing factor 3 subunit 1	NM_001081510	Structural component of the splicing system.	96.5% 0.995	CCTTACCATGCCTACTACCGG	CACTTGGGCTTGAACCTTCTG
EEF1A2	Eukaryotic translation elongation factor 1 alpha 2	BC108110.1	Translation elongation factor activity.	96.8% 0.993	GCAGCCATTGTGGAGATG	ACTTGCCCGCCTTCTGTG
SAA	Serum amyloid A	AF160867.2	Lipid metabolism and transport; immuno-modulatory functions.	94.9% 0.999	CCTGCTGGCCTGCCTGAC	GCTGCCTTCTGAGGACAGAG
Hp	Haptoglobin	NM_001040470	The binding of haemoglobin; immuno-modulatory functions.	101.1% 0.996	CCAAGATGGTCTCCCAGCAT	GTGAGGAGCCATCGTTCAATTG

Table 4.2: Candidate reference genes and APPs genes used for qPCR.

PCR condition		**Thermal profile applied:**	
Buffer (Vivantis)	1X	1st denaturation	2' at 94°C
$MgCl_2$	1.5mM	2nd denaturation	30" at 94 °C
dNTP	0.2mM	Annealing	30" at 56 °C
Primer forward	1 µM	Extension (35 cycles)	45" at 72 °C
Primer reverse	1 µM	Final extension	10' at 72 °C
Taq Polymerase (Vivantis)	0.025 units		

Table 4.3: The amplification condition and thermal profile of qualitative PCR. The reactions were performed in 10 µL final volumes and the fragments were visualized on 1.9 % agarose gel stained with ethidium bromide.

Quantitative reactions were performed in 15 µl of Eva Green mix (BioRad Laboratories) and 400 nM of GAPDH, SDHA, YWHAZ, SF3A1, EEF1A2, ACTB, HMBS, SAA primers and 450 nM of Hp primers (Table 4.2) on iCycler iQ Real Time PCR detection System (BioRad). In order to evaluate the PCR efficiency using a relative standard curve, series of dilution were prepared by performing fourfold serial dilution starting from the pooled sample in triplicate. Each sample was tested in duplicate. No-RT controls were performed by omitting reverse transcription and no template controls were conducted by adding nuclease free water. The thermal profile used (95 °C for 90 s, 50 cycles of 95 °C for 15 s and 60 °C for 60 s; for melting curve construction, 55 °C for 60 s and 80 cycles starting to 55 °C and increasing 0.5 °C each 10 s) was the same for each target gene. The DNA extraction procedures, PCR experiments and analyses were carried out following. The minimum information for publication of quantitative real-time experiments guidelines (MIQE; *Bustin et al., 2009*) was respected to treat samples and perform experiments.

To determine the stability of the selected RGs, two softwares were used: geNorm™ for Microsoft Excel asdescribed by *Vandesompele et al. (2002)* and Normfinder© version 0.953 as reported by *Andersen et al. (2004)*. For both programs raw quantification cycle (Cq) values were converted to relative quantities using the comparative Cq method as described in the geNorm™ manual (http://medgen.ugent.be/~jvdesomp/genorm/).

The two methods generate a measure of reference gene stability, which can be used to rank the RGs. GeNorm™ generates an M value for each gene which is arbitrarily suggested to be lower than 1.5 (with a lower value indicating increased gene stability across samples), and a pairwise stability measure to determine the benefit of adding extra RGs for the normalization process (again with a lower value indicating greater stability of the normalization factor). An arbitrary cut off value of 0.15 indicates acceptable stability of the reference gene combination. NormFinder© generates a stability measure of which lower value indicates increased stability in gene expression and groups samples to allow direct estimation of expression variation, ranking genes according to the similarity of their expression profiles by using a model-based approach.

4.4 RESULTS

Analysis of the gene expression stability over the different tissues by using geNorm™ is presented in Fig. 4.1A; the RGs with the lowest M value indicate the most stable expression and vice versa. SF3A1, HMBS and ACTB were the 3 most stable genes. The M values of these genes were 0.50328 for SF3A1 and HMBS and 0.51633 for ACTB, which indicates good stability. The GAPDH and YWHAZ can be accepted as well, with M values below 1.5. The result indicated EEF1A2 as the worst-M values gene and was therefore excluded from the list of suitable RGs.

NormFinder© identified HMBS as the most stable gene with a stability value of 0.294, followed by ACTB (0.330) and GAPDH (0.427) (Fig. 4.1B). SDHA remained the least stable reference gene as calculated by geNorm™. The software identified HMBS and ACTB as the best combination of two genes, with a stability value f 0.226.

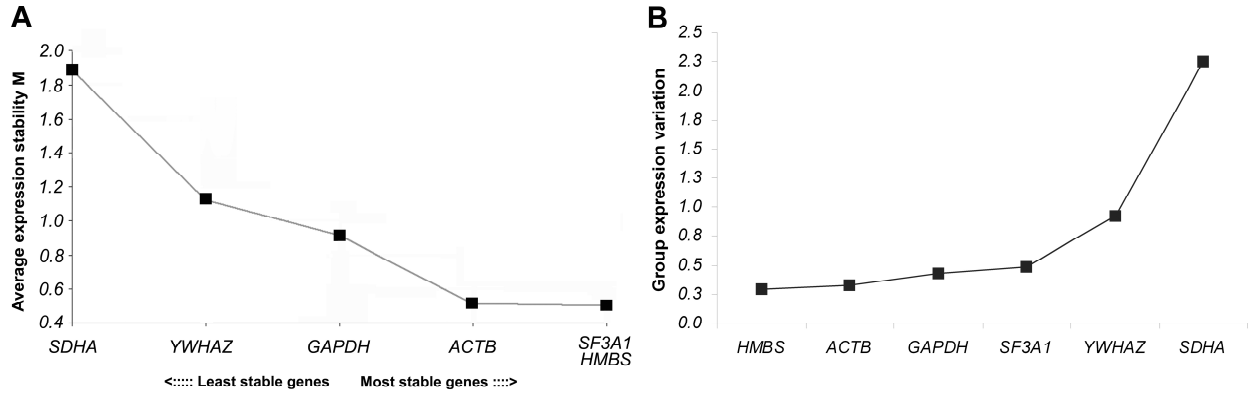

Fig. 4.1. Gene expression stability of candidate RGs. **(A)** Gene expression stability of candidate RGs in bovine selected tissues analyzed by the geNormTM software, which proceeds to the stepwise exclusion of the genes whose relative expression levels are more variable among tissues samples, threshold for eliminating genes as unstable was $M \geq 1.5$. Lower values of M correspond to the most stable genes, hence the most suitable for normalization. **(B)** Gene expression stability of candidate RGs in bovine selected tissues analyzed by the NormFinder© software; the lower value of stability measure indicates increased stability in gene expression.

Results presented in Figs. 4.2 and 4.3 clearly identified SAA and Hp's mRNA in all analyzed tissues. Most of the studies on acute phase proteins are basically focused on their expression during the systemic reaction to inflammation. On the contrary, the present results demonstrated that almost all tissues can locally produce SAA and Hp also in non pathological conditions, even if liver is probably responsible for the production of most of circulating SAA and Hp.

As a whole, extrahepatic production of SAA is higher than that of Hp.

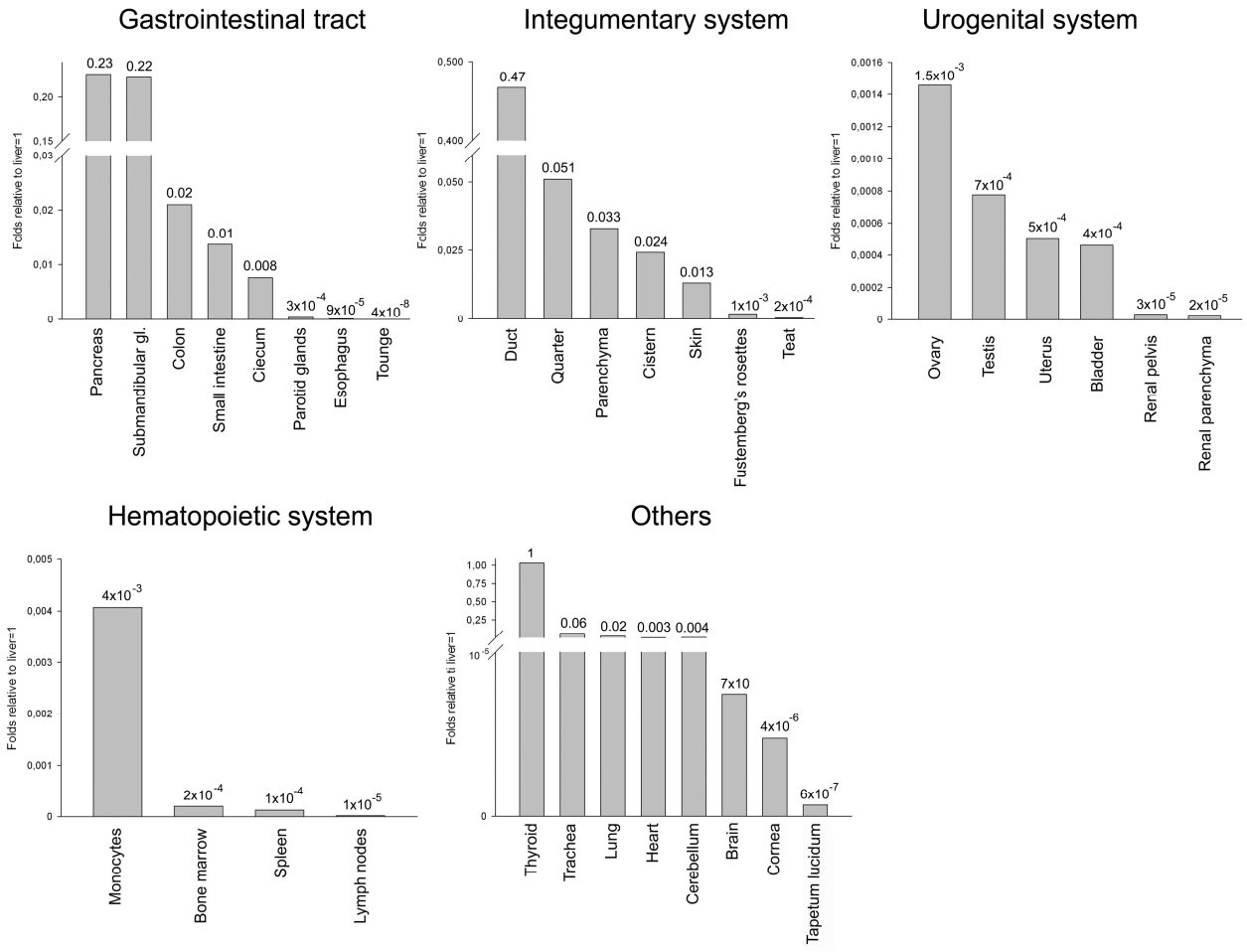

Fig. 4.2. Relative **SAA** gene expression in bovine tissues studied by qPCR. The results were normalized using ACTB, SF3A1 and HMBS as RGs as resulted from geNorm™ analysis. Liver was used as reference sample and data are means of 2 different animals.

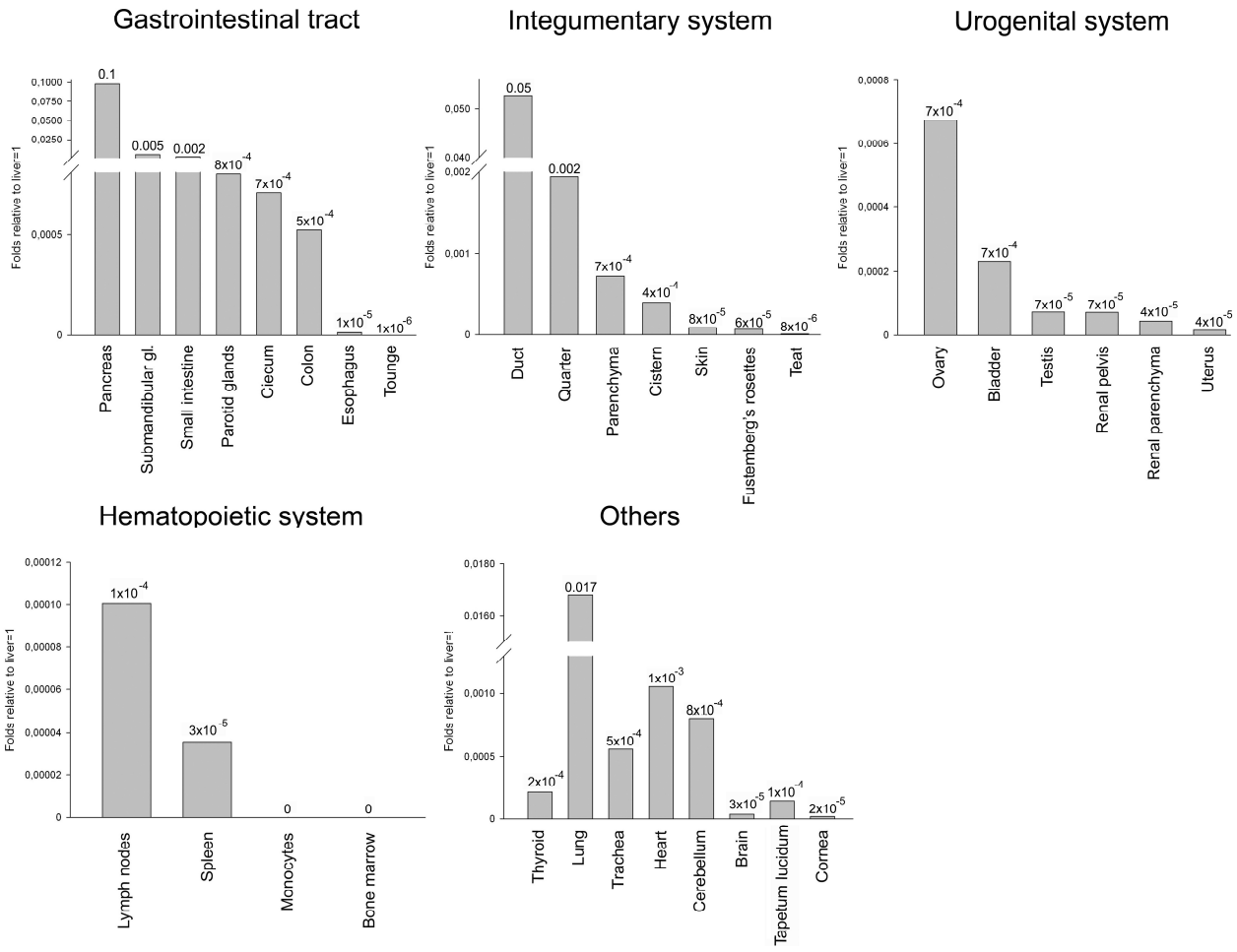

Fig. 4.3. Relative **Hp** gene expression in bovine tissues studied by qPCR. The results were normalized using ACTB, SF3A1 and HMBS as RGs as resulted from geNorm™ analysis. Liver was used as reference sample and data are means of 2 different animals.

4.5 DISCUSSION

The optimal number of reference genes was determined with geNorm™ by means of the pairwise variations (Vn/n + between the sequential normalization factors (NFn and NFn+1) after successive inclusion of less stable RGs. The value of the pairwise variations suggests that the inclusion of a third reference gene contributes to the stability. Fig. 1A presents the evidence that geNorm™ analysis recommended the normalization of RT qPCR by using the ACTB, SF3A1 and HMBS. On the contrary, the high values of V3/4, V4/5 and V5/6 represent the instability of SDHA, YWHAZ and GAPDH, which is also clear in the steep rise presented in the Fig. 4.1A.

NormFinder© model-based approach ranks genes according to the similarity of their expression profiles and generates a stability measure (ρ), which assigns the lower values to the most stable genes.

The GAPDH would be appropriate RGs according to both geNorm™ and NormFinder© analysis, but the matter is controversial; many authors do not consider GAPDH a good reference gene *(Pérez et al., 2008; Spalenza et al., 2010)*, while some others regard GAPDH a stable reference gene *(Hosseini et al., 2010; Robinson et al., 2007)*.

Since pairwise analysis indicated a V2/3 lower than the recommended cut-off point, it was not required the inclusion of an additional RG *(Vandesompele et al., 2002)*. Therefore, the RGs suggested were SF3A1, HMBS and ACTB. Their geometric mean was used for the normalization of the relative quantification data experiments, which were carried out in the final part of the study.

The present study demonstrated that all tissues can locally produce SAA and Hp also in non pathological conditions.

SAA and Hp are expressed in organs communicating with external cavities, such as mammary gland, salivary glands and pancreas. Consistently with a recent report *(Molenaar et al., 2009)*, most of mRNA was found to be expressed by duct cells. These results confirm that mammary gland could constitutively produce both SAA and Hp not only during mastitis *(McDonald et al., 2001; Thielen et al., 2007)*.

The finding that SAA and Hp mRNA are synthesized in the salivary gland, and probably secreted in saliva, is new and parallels what has been previously reported for other acute phase proteins, including LPS-binding protein *(Rahman et al., 2010)* and α1-acid glycoprotein *(Lecchi et al., 2009)*.

Gastro-intestinal expression of SAA and Hp mRNA expression is poor in intestine, pancreas and submandibular glands, in particular if compared with a recent report *(Dilda et al., 2012)*, which identified forestomachs and abomasum as major site of expression of SAA (but not Hp). The present study identified for the first time pancreas as one of the major site of extra-hepatic production of SAA and Hp. The physiological significance of the constitutive expression of SAA and Hp mRNA in pancreas is unknown. We can only speculate about it, on the background of their functions.

SAA is a multifunctional protein involved in a number of processes including cholesterol transport, innate recognition protein of Gram negative and Gram positive bacteria *(Hari-Dass et al., 2005)*, and antimicrobial activity *(Molenaar et al., 2009)*.

Alongside SAA, also Hp has a broad anti-bacterial activity, including hemoglobin (Hb) binding capacity *(Langlois and Delanghe, 1996)*, thus acting as a natural bacteriostat by preventing the utilization of iron by pathogenic bacteria, which require it for their growth. Moreover, on the background of its iron-binding properties, Hp can also

contribute to the anti-oxidant defenses mechanisms of pancreatic cells *(Lenzen, 2008)*. This protective activity may also be fulfilled by SAA due to its inhibitory effect as O_2^- production *(Gatt et al., 1998)*.

With the exception of circulating mononuclear cells, there is no evidence of a cellular phagocyte resident in pancreas. Therefore, pancreas defense against microorganisms moving upstream from the gut relies on pancreatic fluid that flushes the duct. Together with other anti-microbial peptides produced by the pancreas *(Wehkamp et al., 2006)*, we may speculate that SAA and Hp may be conveyed to the mucosa of the pancreatic duct and thus contribute to the defense of the organ. Both SAA and Hp could be therefore part of the host defense mechanism of pancreas, which strongly relies on the anti-bacterial activity of the pancreatic ductal fluid *(Rubinstein et al., 1985)*.

The finding that SAA can be produced by thyroid is new and remarkable. The biological role of SAA expressed in bovine thyroid tissue is not clear. It may also not be ruled out the possibility that local SAA plays a role in the development of bovine AA amyloidosis *(Yamada et al., 2006)* like that has been suggested in humans *(Urieli-Shoval et al.,1998)*.

4.6 CONCLUSIONS

In conclusion, the analysis of expression stability of the 7 chosen RGs using geNorm™ and NormFinder© demonstrated that SDHA and EEF1A2 cannot be used for normalization of the selected 33 tissues. Both algorithms identified SF3A1, HMBS, ACTB and GAPDH to be the most stable RGs in selected samples; the stability ranking was the same, with the exception of GAPDH, which was evaluated more stable than SF3A1 by NormFinder©.

In summary, the present study demonstrated that the two major APPs, namely SAA and Hp, are constitutively expressed in almost all bovine tissues even without the induction of a systemic acute phase response. Taken together, the broad constitutive expression of both these proteins underscores their biological role even in healthy conditions as first line of local immune defense against pathogens. Understanding where acute phase proteins are produced and secreted by is the first step for their proper utilization as biomarkers during diseases.

In general, these results also support the hypothesis that almost every tissue may produce, even in non pathological condition acute phase proteins, and therefore the amount of circulating APP is partially delivered by liver, and partially delivered by tissues.

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Short communication

Widespread expression of SAA and Hp RNA in bovine tissues after evaluation of suitable reference genes

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ABSTRACT

The serum amyloid A (SAA) and haptoglobin (Hp) are the most prominent acute phase proteins (APPs) in cow. Liver mainly produces APPs, but extra hepatic expression has also been demonstrated in some tissues. The major aim of the present study was to assess the constitutive SAA and Hp mRNA expression by quantitative PCR (qPCR) in a wide panel of 33 bovine tissues, including gastrointestinal tract, respiratory system, urogenital system, mammary gland, hematopoietic system, central nervous system, eye, thyroid and heart. Normalization of gene expression in different samples requires reference genes, which are stably expressed. Therefore, seven reference genes were investigated (ACTB, GAPDH, HMBS, SDHA, YWHAZ, SF3A1, EEF1A2) and three genes, namely SF3A1, HMBS and ACTB, were selected after assessing their stability with geNorm™ and NormFinder® softwares.

The qPCR analysis confirmed liver as the principal source of SAA and Hp, but also identified both APPs' mRNA in almost all tissues.

The highest expression rate of SAA was found in thyroid, followed by pancreas and submandibulary gland. Hp mRNA expression was detected at high concentration in pancreas and submandibulary gland.

The present data indicated a widespread expression of SAA and Hp also in non pathological conditions, thus envisaging a possible role as immunomodulatory and protective molecules. To understand where SAA and Hp come from is the prerequisite to their utilization as Acute Phase Reaction biomarkers.

5. *Escherichia coli* lipopolysaccharides and *Staphylococcus aureus* enterotoxin B differentially modulate inflammatory microRNAs in bovine monocytes

5.1 OBJECTIVES AND EXPERIMENTAL DESIGN

This study investigated the effect of *Escherichia coli* lipopolysaccharide (LPS) and *Staphylococcus aureus* enterotoxin B (SEB) on the expression of five miRNAs involved in the inflammatory response, including miR-9, miR-125b, miR-155, miR-146a and miR-223, in bovine $CD14^+$ cells (monocytes).

The experimental design include

1) Purification of bovine monocytes via $CD14^+$ affinity
2) Stimulation of monocytes with Staphylococcus aureus enterotoxin B
3) Assessment through Real Time PCR of miRNA expression.

5.2 INTRODUCTION

MicroRNAs (miRNAs) are small (approximately 22 nucleotides) endogenous non-coding RNA oligonucleotides. They represent an emerging class of small RNA molecules that regulate gene expression by binding to the mRNA of protein-coding genes *(Bartel, 2009)*. There is evidence that miRNAs are also involved in activation of the innate immune response, usually after recognition of pathogens by Toll-like receptors *(Lindsay, 2008)*. Knowledge of the expression patterns of bovine miRNAs is limited to a few tissues and physiological conditions *(Coutinho et al., 2007)*. There is no information about their possible involvement in the regulation of the innate response against invading pathogens.

This study investigated the expression of inflammatory miRNAs after challenge with *Escherichia coli* lipopolysaccharide (LPS) and *Staphylococcus aureus* enterotoxin B (SEB). LPS and SEB were selected to simulate *E. coli* and *S. aureus* infection, respectively. The study was performed on bovine $CD14^+$ cells (monocytes) due to their involvement in the inflammatory reaction during both Gram positive and Gram negative bacterial infection. Enterotoxin B elicits a stronger *in vitro* response in monocytes compared to other Gram negative cell wall components, such as lipoteichoic acid and peptidoglycan *(Skinner et al., 2005)*.

5.3 MATERIALS AND METHODS

Blood was collected for routine disease testing from five clinically healthy cows by venipuncture from the jugular vein. Monocytes ($CD14^+$ cells) were isolated using magnetic activated cellular sorting *(Ceciliani et al., 2007a)*. Cells were cultured in 96 well plates at 2 x 10^5 cells/well and incubated at 37 °C with 5 % CO_2 for 90 min. The medium was substituted with RPMI 1640 containing 100 ng/ml LPS (Sigma-Aldrich, catalogue number L4391) or 100 ng/ml SEB (Sigma-Aldrich, catalogue number S4881) for 14 h. The incubation time was selected following experiments using human monocytes *(Bazzoni et al., 2009)*. Cells incubated with RPMI 1640 were used as negative controls. Our study used five miRNAs (miR-9, miR-125b, miR-155, miR-146a and miR-223) previously identified as being involved in the innate response against pathogens in humans and rodents *(Lindsay, 2008)*. Since miRNA sequences are conserved between these two species, we used commercial oligonucleotide primers and TaqMan probes for human mature miRNAs (Table 5.1). Total RNA was extracted with mirVana miRNA Isolation Kit (Ambion). Reverse transcriptase (RT) reactions were performed with stem-loop primers specific for each miRNA (TaqMan MicroRNA Reverse Transcription Kit; Applied Biosystems) and amplified by Real Time PCR with the corresponding Human TaqMan MicroRNA Assays kit (Applied Biosystems). Relative expression of each miRNA was calculated using the comparative Δ–Δ Ct method; miR-16 was selected as an endogenous control, since there are no reports that this miRNA is modified in response to immune modulators *(Lindsay, 2008)*. Statistical analyses were performed using PASW 18.0 for Windows (SPSS). Descriptive statistics are expressed as the mean ± standard deviation and normality of data distribution was assessed using the Shapiro-Wilk test.

Since the data were normally distributed, a univariate analysis of variance was applied to compare means in different experimental groups.

miRNA	Sequences
Hsa miR-16	5'-UAGCAGCACGUAAAUAUUGGCG-3'
Hsa miR-125b	5'-UCCCUGAGACCCUAACUUGUGA-3'
Hsa miR-146a	5'-UGAGAACUGAAUUCCAUGGGUU-3'
Hsa miR-155	5'-UUAAUGCUAAUCGUGAUAGGGG-3'
Hsa miR-223	5'-UGUCAGUUUGUCAAAUACCCC-3'
Hsa miR-9	5'-UCUUUGGUUAUCUAGCUGUAUGA-3'

Table 5.1: Sequences of miRNAs used in this study. miRNA sequences were obtained from miRBase (http://microrna.sanger.ac.uk) and their identity with the corresponding human homologous was confirmed by BLAST alignment (http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi).

5.4 RESULTS

As shown in Fig. 1, both LPS and SEB differentially modulated the expression of four miRNAs (miR-125b, miR-155, miR-146a and miR-223). In particular, stimulation with SEB down-regulated miR-155, miR-125b and miR-223.

In agreement with previous reports using human and rodent monocytes *(Bazzoni et al., 2009)*, stimulation of bovine monocytes with LPS up-regulated both miR-155 and miR-146a.

Quantitative RT-PCR experiments have demonstrated that miR-9 is poorly expressed in bovine monocytes.

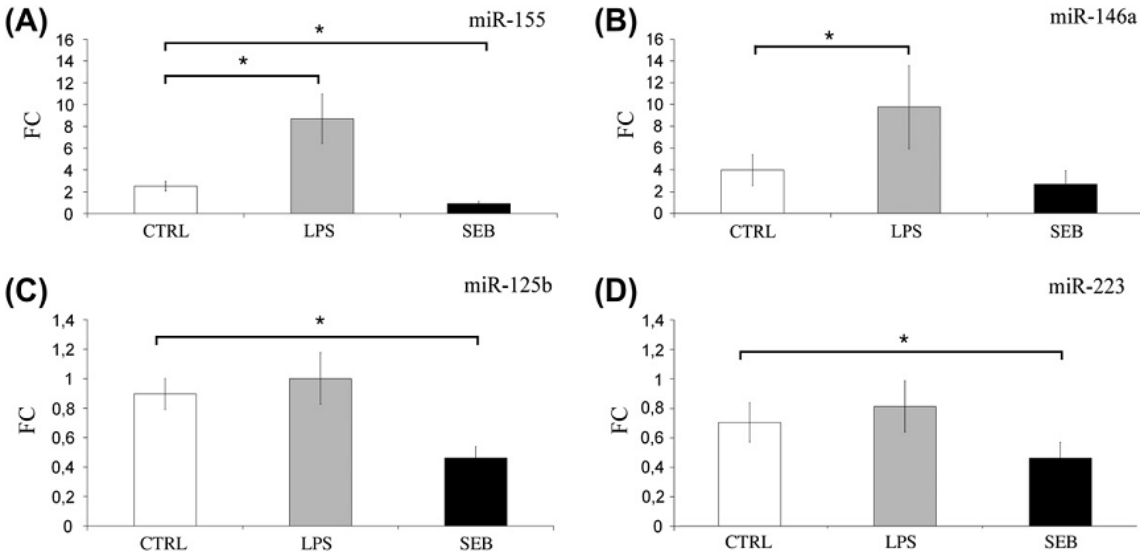

Fig. 5.1.: *Staphylococcus aureus* enterotoxin B (SEB) and *Escherichia coli* lipopolysaccharides (LPS) differentially regulate miRNA expression in isolated bovine monocytes. Bovine monocytes were cultured in the presence of LPS (grey histograms) and SEB (black histograms). White histograms represent negative controls (CTRL). Bars indicate standard errors of the mean. Statistical significance was declared for $P \leq 0.05$ (*). Data shown are means of five experiments. The results were expressed as fold change (FC) *(Bazzoni et al., 2009)*.

5.5 DISCUSSION

The role of miR-155 is to up-regulate pro-inflammatory cytokines, including tumor necrosis factor a (TNF-α) and interleukin-6, in response to bacterial infection *(Bazzoni et al., 2009)*. Given the high degree of homology between bovine and human miRNAs, we speculate that, by down-regulating miR-155, SEB might also down-regulate TNF-α, as reported during *S. aureus* mastitis *(Bannerman, 2009)*. This hypothesis has to be confirmed by loss of function and over-expression studies that, given the limited knowledge of bovine inflammatory pathways, cannot be carried out in this species at this stage. Stimulation of bovine monocytes with SEB also down-regulated both miR-125b and miR-223, consistent with an anti-inflammatory role.

Given the pro-inflammatory activity of miR-155, up-regulation of this microRNA may amplify the pro-inflammatory loop during the first phase of the innate-immune reaction, consistent with the acute inflammatory reaction that follows *E. coli* infection. Conversely, miR-146a has been identified as a molecule preventing an over-stimulated inflammatory state (*Nahid et al., 2009*). Neither miR-223 nor miR-125b were over-expressed after challenging monocytes with LPS. miR-9 results' suggesting there that there is a difference in the expression pattern of this miRNA in cattle compared to humans.

In humans, miR-146a and miR-223 recently have been identified as specific and sensitive biomarkers for sepsis *(Wang et al., 2010)* and have been detected in peripheral blood mononuclear cells and plasma microvesicles *(Hunter et al., 2008)*. In cattle, miR-223 and miR-125b have been identified in milk-derived microvesicles *(Hata et al., 2010)*.

5.6 CONCLUSIONS

The miRNAs identified in this study may play a role in regulating the inflammatory response against pathogens and are modified during inflammatory response and are suitable candidate diagnostic markers.

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Short Communication

Escherichia coli lipopolysaccharides and *Staphylococcus aureus* enterotoxin B differentially modulate inflammatory microRNAs in bovine monocytes

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ABSTRACT

MicroRNAs (miRNAs) are a family of regulatory molecules involved in many physiological processes, including activation of cells of the immune system. This study investigated the effect of *Escherichia coli* lipopolysaccharide (LPS) and *Staphylococcus aureus* enterotoxin B (SEB) on the expression of five miRNAs involved in the inflammatory response, including miR-9, miR-125b, miR-155, miR-146a and miR-223, in bovine $CD14^+$ cells (monocytes). Incubation of monocytes with SEB induced down-regulation of miR-155, miR-223 and miR-125b, but not the anti-inflammatory miRNA miR-146a. Conversely, incubation with LPS upregulated both miR-155 and miR-146a. In vitro incubation of isolated $CD14^+$ bovine monocytes with LPS and SEB elicited different and opposite expression of miRNAs reportedly involved in inflammatory reactions.

6. Development of a sensitive ELISA assay to detect AGP and SAA

6.1 OBJECTIVES AND EXPERIMENTAL DESIGN

The aim of this study was to investigate and to obtain more information on the concentration of α1-Acid glycoprotein (AGP) and Serum Amyloid A (SAA) In particular, the present work has want developed of an ELISA test for the quantification of AGP and SAA in cattle and goat serum.

The study was divided into two parts:

1) Development of a sensitive ELISA assay to detect AGP;

2) Development of a sensitive ELISA assay to detect SAA.

6.2 DESCRIPTION OF SAMPLES

In this study we analyzed the following including:

- ***Bovine samples for AGP assay***:

Healthy animals:

- 13 calves sera;
- 1 pool made by the 13 different sera of healthy animals.

Pathological animals:

- 9 calves sera with diarrhea;
- 1 pool made with serum of animal that presented high values of APPs.

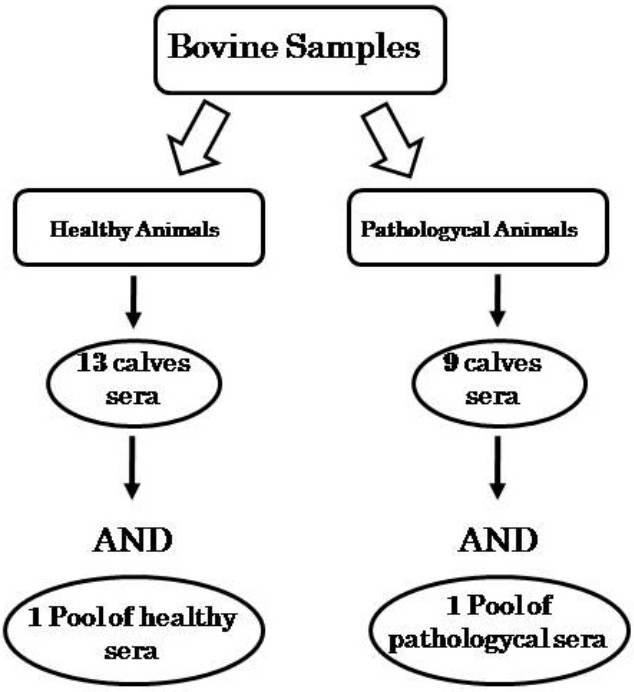


Fig. 6.1: Diagram of bovine samples analyzed in AGP assay.

- ***Goat samples for AGP assay***:

Healthy animals:

- 22 goats sera;
- 1 pool made by 6 different sera of healthy animals.

Pathological animals:

- 68 goats sera with different diseases (17 with CAEV, 16 with brucellosis, 35 with intramammary experimental infection by *E. coli*);
- 1 pool made by 6 different sera of pathological animals.

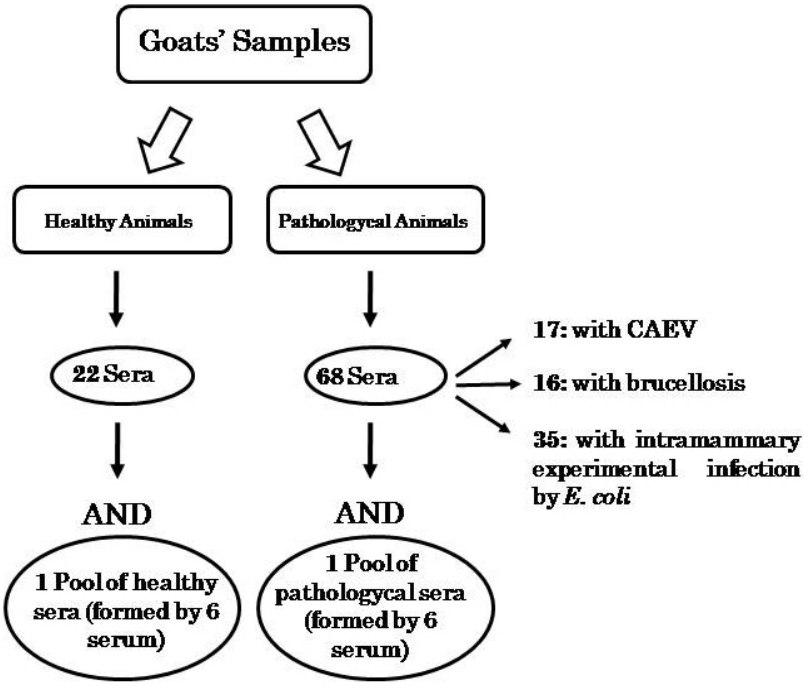


Fig. 6.2: Diagram of goat samples analyzed in AGP assay.

- ***Bovine samples for SAA assay***:

Healthy animals:

- 4 calfs serum;
- 2 bovine healthy sera.

Pathological animals:

- 4 calf serum with diarrhea;
- 4 bovine sera with different diseases (colic, mastitis, physical deterioration, infection).

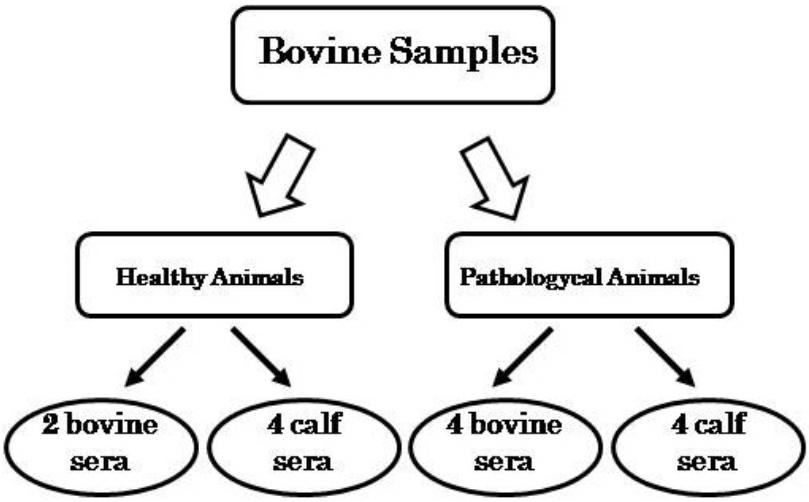


Fig. 6.3: Diagram of bovine samples analyzed in SAA assay.

- ***Goat samples for SAA assay***:

Healthy animals:

- 39 goats sera;

Pathological animals:

- 40 goats sera with different diseases (3 with CAEV, 3 with brucellosis, 2 with non-specific diseases, 35 with intramammary experimental infection by *E. coli*);
- 1 pool made by 20 different sera of goats experimental injected with Turpentine oil.

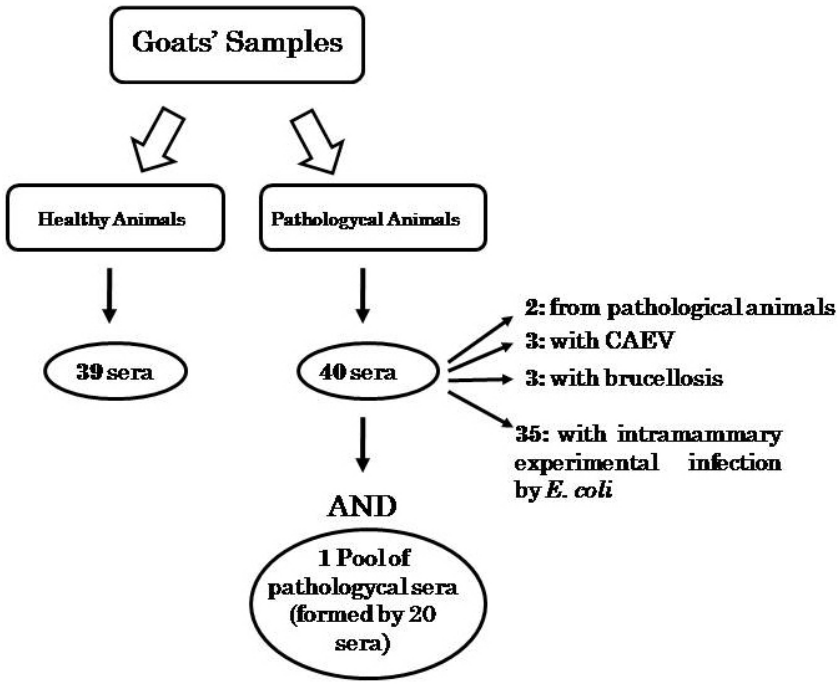


Fig. 6.4: Diagram of goat samples analyzed in SAA assay.

6.3 BRIEF DESCRIPTION OF THE MAIN DISEASES PRESENT IN SAMPLES

6.3.1 Caprine arthritis-encephalitis virus (CAEV)

Caprine arthritis-encephalitis virus (CAEV) and ovine *Maedi-visna virus* (MVV) are members of the small ruminant lentiviruses (SRLVs) group in the retroviridae family infecting goats and sheep worldwide *(Pasick, 1998; Pepin et al., 1998)*. CAEV was first isolated from the joint of an arthritic goat and simultaneously from the brain of an encephalitic kid *(Crawford et al., 1980; Narayan et al., 1980)*. The virus genome was cloned, fully sequenced and the genes coding the viral proteins characterized *(Saltarelli et al., 1990; Saltarelli et al., 1994)*. The genomic organization of SRLVs is typical of lentiviruses: positive sense RNA dimmers of approximately 9 kb in size which consist of long terminal repeats (LTRs), gag (group specific antigens), pol (polymerase) env (envelope) genes in addition to a number of regulatory genes. The gag and pol genes are relatively well conserved among SRLVs, which makes them ideal targets for PCR primer design *(Pepin et al., 1998)*. Small ruminant lentiviruses (SRLVs), CAEV and VMV, have been assigned to five distinct genetic groups (A to E) that are subdivided into numerous subtypes *(Reina et al., 2010; Shah et al., 2004)*. The groups A and B are predominant and refer to VMV-like and CAEV-like strains, respectively. The underlying mechanisms and consequences associated with SRLV adaptation to new hosts following interspecies transmission are poorly explored and understood. Since SRLV strains differ in their biological properties, the outcome of the infection could vary widely. For instance, some group A viruses are highly neurovirulent while group B viruses seem to have a propensity to induce arthritis *(Glaria et al., 2009; Oskarsson et al., 2007)*. Recently, a

significant higher rate of lactogenic transmission has been reported for VMV-like viruses in goats coinfected with SRLV groups A and B *(Pisoni et al., 2010)*. Several lines of evidence indicate that host determinants may also influence the outcome of SRLV infection.

Common clinical signs caused by SRLV infections include neurological disorders, dyspnoea, emaciation, mastitis and arthritis *(Pepin et al., 1998; Narayan and Clements, 1989; Alvarez et al., 2006)*. Molecular-epidemiological evidence suggest that SRLVs can transmit between sheep and goats under favorable conditions *(Shah et al 2004; Pisoni et al 2005)*. In Canada, SRLV infections are widespread in small ruminants and have been associated principally with lung and mammary lesions in sheep and arthritis and emaciation in goats *(Arsenault et al., 2003; Simard and Morley, 1991)*.

CAEV is an ungulate lentivirus that causes severe, progressive central nervous system disorders, arthritis, mastitis and eventually death of infected animals *(Cheevers and McGuire, 1988)*. CAEV is responsible of a severe, systemic, inflammatory status. It is therefore possible, but still not thoroughly investigated, that CAEV is also accompanied by an acute-phase reaction since, notwithstanding its name, the so-called "acute-phase response" may occur in both acute and chronic systemic inflammation *(Gabay and Kushner, 1999)*. CAEV-induced arthritis has been already studied as a model of inflammatory disease which may alter glycan micro-heterogeneity of plasma proteins *(McCulloch et al., 1995)*.

Chronic arthritis is considered a very reliable model for investigating such post-translational modifications during inflammatory reactions. AGP oligosaccharides from human patients with rheumatoid arthritis presented a significant increase in

fucosylation and tri-sialylated N-glycans expression *(Havenaar et al., 1998)*. AGP has been found at high concentration in inflamed articulations, but it has been suggested that its increasing in synovial fluid is of hepatic origin *(Havenaar et al., 1997)*.

6.3.2 Brucellosis

The WHO considers brucellosis to be a neglected zoonosis because, despite its widespread distribution and effects on multiple species, it is not prioritised by national and international health systems *(World Health Organisation (WHO): The Control of Neglected Zoonotic Diseases)*. It is caused by gram-negative bacteria of the genus *Brucella* which show strong host preference *(Corbel et al., 1984)*. The species of *Brucella* which infect livestock and their primary hosts are *B. melitensis* (sheep and goats), *B. abortus* (cattle), *B.suis* (pigs) and *B. ovis* (sheep) *(Meyer, 1990; Crawford, 1990)*. Brucellosis decreases productivity of infected livestock by causing abortions, reducing fertility and decreasing milk yield, resulting in substantial economic losses *(Corbel, 1988; Nicoletti, 1980)*. Brucellosis, especially caused by *B. melitensis*, remains one of the most common zoonotic diseases worldwide with more than 500,000 human cases reported annually *(Seleem et al., 2010)*. With the exception of *B. ovis* and *B. neotomae*, all *Brucella* species can cause infections in humans. New Brucella species pathogenic for humans – *B. ceti* and *B. pinnipedialis* – have recently been discovered in marine mammals *(Foster et al., 2007)*. Infection is transmitted to humans though direct contact with the infected animals or by consuming infected milk or fresh cheese *(Seleem et al., 2010)*. *B. melitensis* has 3 biovars (1-3), highly pathogenic for humans *(Pappas et al., 2005)*. It is endemic in sheep and goats in most countries of the Mediterranean basin, the

Middle East, Central Asia (*Omer et al., 2000, Radostits et al., 2000)*, with only North America, North Europe, South-East Asia, and Oceania being spared *(FAO/OIE/WHO, 1995 Animal Health Yearbook).*

Several Middle Eastern and central Asian countries have reported an increase in the incidence of human brucellosis and the appearance of new foci *(Pappas et al., 2006)*. In Egypt, brucellosis is endemic among humans and domestic ruminants *(Refai, 2002)*, and it has recently been found that catfish in the Nile Delta region can be naturally infected with *Brucella melitensis (El-Tras et al., 2009).*

Animal brucellosis poses a barrier to trade of animals and animal products between countries and causes considerable economic losses due to abortion and fertility problems to the sheep and goat industry *(Benkirane, 2006, Aldomy et al., 2009)*. Control measures are based on strict hygiene and vaccination programs. Serological test, identification of the agent by culture and Polymerase chain reaction (PCR) test are the most common techniques that are used for brucellosis diagnosis *(OIE, "Terrestrial manual, Bovine brucellosis", 2009).*

6.3.3 Experimentally induced inflammation by Turpentine oil in goats

These samples derived from six clinically healthy Murciano-Granadina goats (mean age: 7 years; age range: 6-9 years). Turpentine oil was injected once (5 ml) subcutaneously above the ribs. Blood samples (5 ml) were collected from the jugular vein in plain tubes before injection (day 0) and at 1, 2, 3, 4, 5, and 8 days after injection. Blood in plain tubes was allowed to clot for 2 h and then centrifuged. The obtained serum was frozen (-20°C) until analysis *(Gonzàlez et al., 2008)*. This part of experiment was carried out in cooperation with the University of Murcia – Spain.

6.3.4 Experimentally induced intramammary inflammation by *E.coli* in goats

Six clinically healthy goats were used in this study. *E. coli* was injected once at intramammary level. Blood samples were collected from the jugular vein in plain tubes before injection (time 0) and at 4h, 8h, 24h, 48h, 72h, and 7 days after injection. This part of experiment was carried out in cooperation with the University of Satara Zagora – Bulgaria.

6.4 Development of a highly sensitive sandwich ELISA to quantify the goat and bovine AGP in serum

6.4.1 OBJECTIVES

The aim of the present study was develop of an ELISA test for the quantification of α1-Acid glycoprotein (AGP) in cattle and goat serum.

The study was divided into two parts:

- Immunization of rabbits with pure AGP for the stimulation of the immune system and therefore production of antibody;
- development of ELISA test for the quantification of AGP.

6.4.2 MATERIAL AND METHODS

Production of rabbit polyclonal antibodies

Polyclonal antibodies were produced using a protocol based on the one described by the UC Bekerley Animal Care and Use Comitee (2006), with some modifications. In brief, two New Zealand rabbits three mouth old were immunized with 250 µg of pure bovine AGP emulsified in Freund's complete adjuvant (first immunization) or Freund's incomplete adjuvant (subsequent immunizations) by intradermal injections every two weeks for four weeks.

One week after the last immunization, blood samples were collected at the marginal vein of the ear. The blood is was taken in tubes with lithium-heparin and the quantity that was taken it was calculated on animal's weight (6 ml/Kg). This blood was allowed to clot, centrifuged at 2000 g for 10 min and serum was separated and kept frozen at -20 °C until purification. IgG content of the serum collected was purified using affinity chromatography with a G-protein HiTrap column (GE Healthcare Life Sciences, Uppsala, Sweden) attached to a FPLC system (GE Healthcare Life Sciences, Uppsala, Sweden). Plasma was centrifuged at 6480 g for 5 min at 4 °C to remove impurity. IgG obtained after the purification were concentrated by Centricon Amicon Ultracel-30 K (Millipore), centrifuging again at 7500 g for 10 min at 4 °C. The final volume of purified IgG was about 1 ml.

The purity of the purified antibodies was assessed by 12 % SDS-PAGE; their concentration was obtained reading the absorbance at 280 nm by spectrophotometer (JASCO V-530, UV/VIS spectrophotometer). Subsequently, before to start the labeling with biotin, antibodies were dialyzed using NAP-10 Columns (GE Healthcare Life Sciences, Uppsala, Sweden), following manufacturer's instructions, against PBS pH 7.2-8, the final concentration being within 1-10 mg/ml in a volume of 0.5-2 ml.

An aliquot of antibodies were labeled with biotin using a commercial kit (No weigh Sulfo-NHS-biotin, Pierce, Thermo Fisher Scientific, Whaltman, MA, USA) following manufacturer's instructions, as follows.

1	Reform the biotin contained in the tube to obtain a solution 10mM
2	Calculate the volume of biotin solution necessary for the labeling: Calculate Ab's mol (Y). considering that MW of IgG is equal to 160.000 g/mol: (X mg Ab x 1000)/160.000 = Y mol di Ab Calculate mmol of biotin (Z): ml Ab x (mg Ab/ml Ab) x (mmol Ab/mg Ab) x (20 mmol biotin/mmol Ab) = Z mmoli biotin Convert mmol of biotin µl: Biotin mol x (10^6 µl/L) x (L/10 mmol) = biotin µl
3	Take the necessary volume of biotin solution and incubate at room temperature for 30 min
4	At the end of the incubation, to remove the unbound biotin, make a dialysis with Nap-10 (GE Healthcare Life Sciences, Uppsala, Sweden) using TSA buffer (Tris-HCl pH 7.5 + NaCl 150mM + NaN_3 0.02

Table 6.4.2.1. Summary of Sulfa-NHS-Biotin's protocol (Pierce, Thermo Fisher Scientific, Whaltman, MA, USA).

Western blot analysis

Samples tested were separated by 12 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotted onto nitrocellulose membranes. The membranes were immunolabelled for the presence of AGP using specific antibodies (that we had produced) and immunoreactive bands were visualised by enhanced

chemiluminescence (ECL) using Immobilon Western Chemiluminescence substrate (Millipore).

Enzyme-linked immunosorbent assay (ELISA)

Instrumentation and Materials

- Plates agitator;
- Plates washer;
- spectrophotometer (JASCO V-530, UV/VIS spectrophotometer);
- Plates (NUNC-immuno plate, made in Denmark);
- Coating Buffer (bicarbonate/carbonate 100 mM pH 9.6: 3.03 g Na_2CO_3; 6 g $NaHCO_3$; 1000 ml H_2O Mq.)
- Roti Block (Carl Roth GmbH & Co. KG, Karlsruhe, Germany);
- PBST (PBS + Tween 20 (Sigma-Aldrich Corp., St. Louis, MO, USA) 0,1%);
- Streptavidin-Peroxidase-Polymer-Ultrasensitive (HRP) (Polymer, Ultrasensitive, Sigma-Aldrich Corp., St. Louis, MO, USA);
- Substrate solution (sodium citrate 0,1 M, citric acid 0,1 M, ABTS (Sigma-Aldrich Corp., St. Louis, MO, USA) and H_2O_2.

The development, optimization and validation of this assay were divided in several steps:

1.~Selection of the suitable antibody for the competitive ELISA assay: the response of each of the two different antibodies to a competitive assay was assessed. In brief, Polystyrene 96 wells microtiter plates (Nunc-Immunoplate Maxisorp, Nunc, Denmark) were coated over-night at 4 °C with 100 µl/well of a specific bovine polyclonal antibody (2.5 µg/ml) in carbonate/bicarbonate buffer, pH 9.6. Plates were washed four times in phosphate buffer saline containing 0.1% (v/v) Tween-20 (PBS-T). 250 µl/well of blocking solution RotiBlock 1X (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were dispensed and the plates were incubated over-night at 4 °C. Subsequently, plates were washed four times in phosphate buffer saline containing 0.1% (v/v) Tween-20 (PBS-T). One hundred microliter of a series of double dilutions standard (pure bovine AGP) solution ranging from 500 to 7.8125 ng per well was added to one row along with dilution buffer (RotiBlock 1X), sample (conveniently diluted in blocking solution 1X) and blank, were added in duplicate to the appropriate well, and the plates were incubated 1 h at room temperature in a flutter. Afterward, plates were washed four times in phosphate buffer saline containing 0.1% (v/v) Tween-20. One hundred microliter of a specific bovine polyclonal biotinylated antibody (2.5 µg/ml) opportunely diluted in RotiBlock 1X were dispensed and the plates were incubated 1 h at room temperature in a flutter. After washing, 1:1000 streptavidin-perxidase polymer-ultrasensitive (Sigma-Aldrich Corp., St. Louis, MO, USA) was incubated for 1 hour at room temperature in a flutter in the dark and the reaction was measured by the addition of ABTS substrate (Sigma-Aldrich Corp., St. Louis, MO, USA) and 405 nm kinetic reading every 5 minutes for 20-55 minutes in a microplate spectrophotometer reader (Labsystem Multiskan MS).

2.~Optimization of coating antibody and biotinilated antibody concentrations: 150, 200, 250 and 300 ng of antibody per well in combination with concentrations with standard

curve obtained with pure boAGP and 200, 250 and 300 ng of biotinilated antibody per well were tested. The combination of minimum concentration of reactants and good regression fit was chosen.

3.~Standard curve: Several dilutions of boAGP standard were tested to build a standard curve that would allow a wide assay range and avoid hook effects, showing a good fit to regression analysis.

4.~Optimal sample dilution: a range between 1:4 to 1:256 of two bovine sera pools (or two goat sera pools), one with presumably high and one with presumably low concentration of AGP were tested to determine the concentration than best fitted the range of the standard curve. Each pool was prepared by mixing serum samples from different animals with similar AGP concentrations.

Once optimized the assay, the analytical performance was validated.

Statistical Analysis

Regression analysis and One Site Total non-parametric test was used by the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com"). t Test of significance (significance level was settled at $p < 0.05$) was used to compare values of AGP in healthy and diseased bovine, or goat, and between and within-run CVs and detection limits were carried out by standard descriptive statistical methods by using Microsoft Excel 2000 (Microsoft Corporation. USA).

Analytical validation of the assay

Assessment of precision. To calculate within-assay precision, AGP content of pools was measured 6 times in the same analytical run, while between-assay precision was determined by measuring the same pools on 6 days within a 7-day period.

The intra-assay precision was calculated through calculating the coefficient of variation (CV %),

$$CV\% = (STDEV\ Abs/Average\ Abs)*100$$

Assessment of the accuracy. In order to perform this type of analysis is necessary to assess linearity. The linearity is determined calculating the expected concentrations, based on the calibration curve, of the "high" pool and the "low" pool, suitably diluted by using assay blocking buffer (and the content of AGP was measured for each dilution), with the observed concentrations. The different dilutions were evaluated by linear regression analysis, comparing observed values with expected values. Then, it was determined the R^2 coefficient.

Evaluation of sensitivity. This evaluation was assessed by the determination of 2 different parameters: analytical limit of detection and limit of quantification. The first is done through the analytical assay, or rather evaluating different white and analyzing them as if they were samples. With the results of absorbance obtained it was calculated concentration, always based on the standard curve. With the concentrations obtained in this way was determined the limit of detection that is equal to:

Average+(STDEV *3)

Furthermore, were calculated lower limit of quantification (LLOQ), which is the lowest concentration of analyte that can be quantified with a precision and accuracy acceptable, and the upper limit of quantification (ULOQ), which represents the highest concentration of analyte that can be quantified with a precision and accuracy acceptable. In order to calculate these values have been made in replicated 6 calibration curves; the absorbance of one of these was taken as a reference to calculate the concentrations of the other 5. Afterwards, for each dilution CV was determined (as described above).

6.4.3 RESULTS

Bovine assay

Western blot analysis

In the first part of this study we have obtained two antibodies able to recognize specifically bovine AGP (Fig.6.4.3.1).

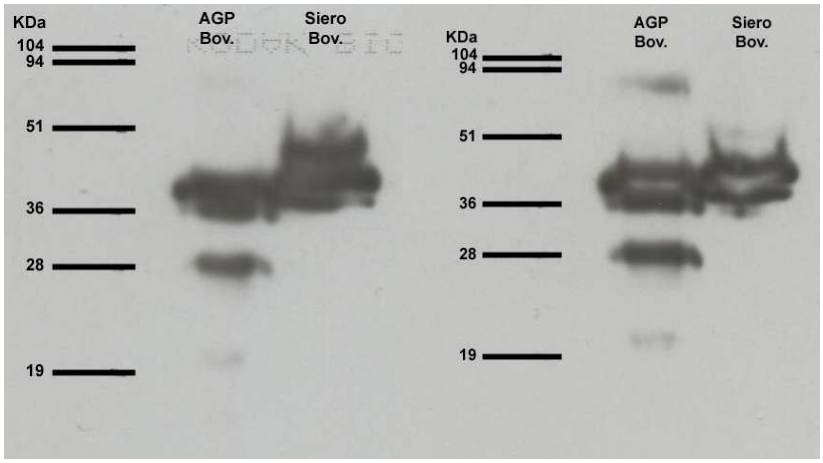


Fig.6.4.3.1: Western Blot for analyze the specificity of antibodies. A) first lane: 100 ng of purified bovine AGP; second lane: 1µl of bovine serum; primary antibody anti-AGP 1:2000 (rabbit one); secondary antibody: peroxidate anti-rabbit 1:2000. B) first lane: 100 ng of pure bovine AGP; second lane: 1µl of bovine serum; primary antibody anti-AGP 1:2000 (rabbit two); secondary antibody: peroxidate anti-rabbit 1:2000. Detection by chemiluminescence (ECL, Millipore).

For the test ELISA to detect bovine AGP the best antibodies concentration to use for the coating was identified to be 250 ng per well and we used the antibody produced in rabbit

2; for biotinylated antibody the best concentration to use was identified to be the same of coating, but since we could not use the same antibodies, we used the one produced in rabbit 1.

Regarding the calibration curve, made by using purified AGP, the protein concentration ranged from 5 µg/ml to 0.078125 µg/ml to be able to locate the AGP in an accurate manner without encountering the so-called "hook effect". T several experiments carried out also demonstrated that the ideal dilution of samples of bovine plasma was 1:50, since at this dilution the absorbance values that are obtained falled in the middle of standard curve.

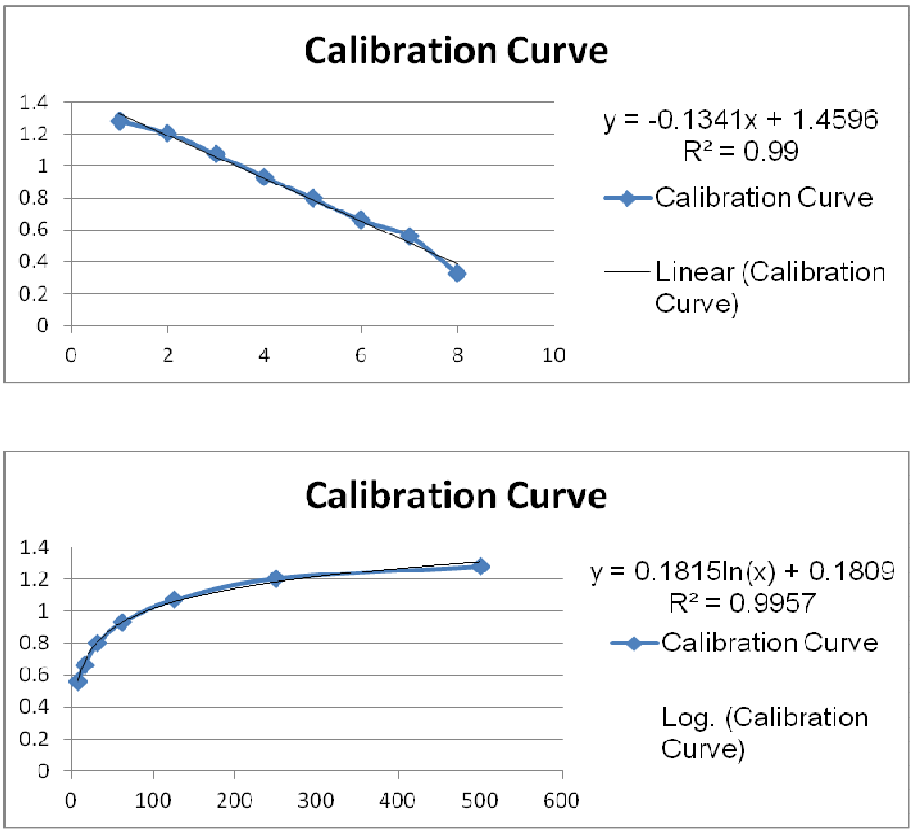


Fig.6.4.3.2: the calibration curve, constructed using pure AGP using the following concentrations of protein: 5, 2.5, 1.25, 0.625, 0.3125, 0.15626, 0.078125 µg/ml.

To validate the test it was necessary to create a pool with sera of healthy animals, so as to obtain the pool with low values of AGP. The pool was prepared out by mixing equal volumes of samples. For high values of AGP was not obtained a pool because we still had left only the serum of one animal that presented very high levels of protein for certain.

The analytical validation of the assay was thus carried out as follows:

Assessment of precision.

In this test, for the serum pool with high values of AGP CV was of 12 %, while for the serum pool with low values of AGP it was obtained a CV of 13 %.

These values are acceptable for both pools and high concentration sample, since for a validation of an ELISA assay an INTRA-assay precision CV lower 15 % and acceptable values below than 20 % are regarded as "good" results.

INTER-assay accuracy was then calculated both for the pool "high" that for the pool "low". CV values obtained were to 14 % and therefore "in range", since to be defined as a "good value" to validate the test, it must be lower than 15 %.

Assessment of the accuracy.

Validation of the accuracy for "high" pool resulted in a R^2 of 0.966 and for the 'low' pool a R^2 of 0.984. Both these values confirm that the test has an acceptable accuracy, given that they are close to one. In order assess the accuracy, the recovery level was evaluated as well. Therefore, defined concentrations of pure AGP (starting from 5 µg/ml and going on with serial dilutions of 1:2) were added to both pools. Data obtained were compared

with expected values and a percentage evaluation was made. All the values obtained were close to 100 %, indicating once again that the test has a good accuracy.

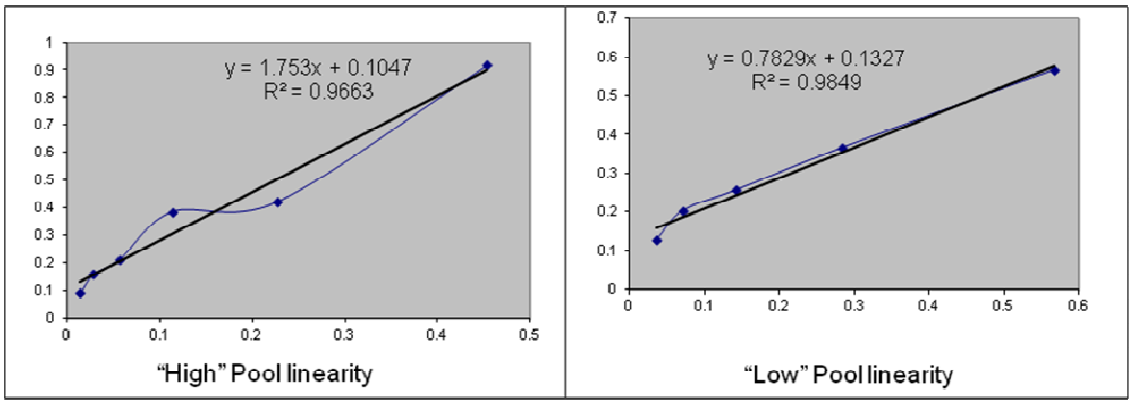


Fig.6.4.3.3: graphic representation of "High" pool and "Low" pool linearity in bovine test.

Evaluation of sensitivity. The limit of detection was 0.03 µg/ml.

Upper limit of quantification (ULOQ) represents the concentration that among the highest values of the calibration curve has a coefficient of variation (CV) upper than 20 %. In this assay ULOQ was 2.5 µg/ml; lower limit of quantification (LLOQ) is the concentration that among the lowest values of the calibration curve has a CV upper than 20 %.,In this assay corresponds to the limit of detection (0.03 µg/ml), because the CV values of the lower values of the calibration curve are all less than 20 %.

The ability of the assay to distinguish between healthy and diseased animals was tested in 13 serum from healthy calves and 19 from cow/calves with disease (9 calves with diarrhea and 10 cows with different disease) were analyzed (Fig. 6.4.3.4).

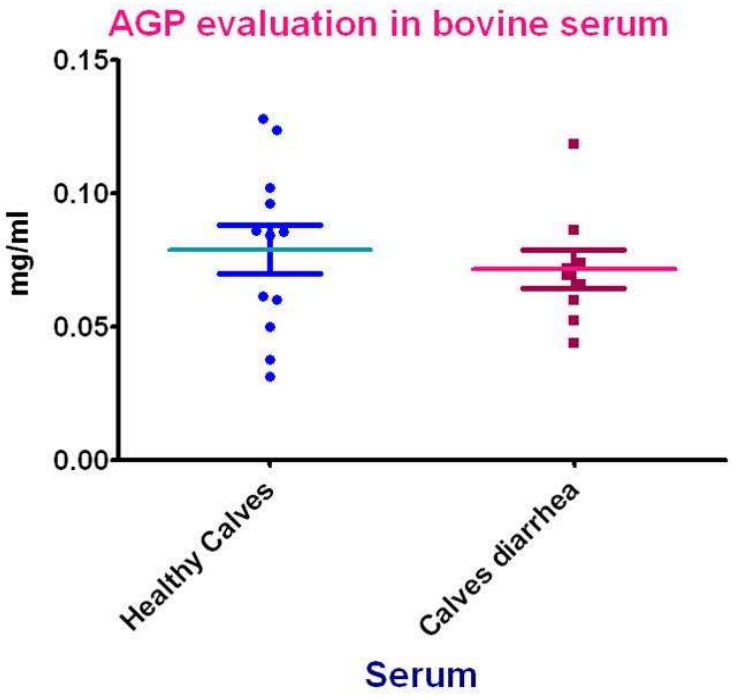


Fig.6.4.3.4: graphic representation of bovine serum evaluation.

In our study, no statistically significant differences in the levels of AGP between healthy calves and calves with diarrhea were detected with our ELISA test.

Goat assay

Western blot analysis

In the second part of this study the cross-reactivity of the bovine antibodies with goat AGP was assessed by Western blotting (Fig. 6.4.3.5).

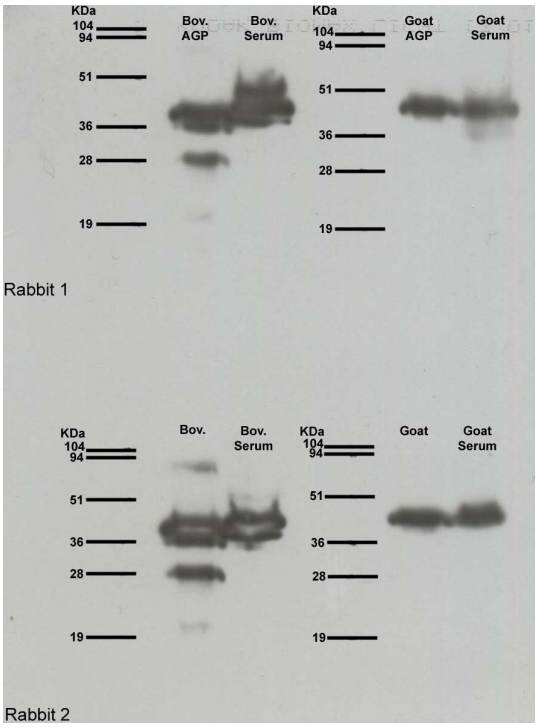


Fig.6.4.3.5: Western Blot for analyze the cross-reactivity of antibodies. A) First lane: 100 ng of pure bovine AGP, second lane: 1µl of bovine serum; primary antibody anti-AGP 1:2000 (rabbit one); secondary antibody: peroxidate anti-rabbit 1:2000. B) First lane: 100 ng of pure goat AGP, second lane: 1µl of goat serum; primary antibody anti-AGP 1:2000 (rabbit one); secondary antibody: peroxidate anti-rabbit 1:2000.C) First lane: 100 ng of pure bovine AGP, second lane: 1µl of bovine serum; primary antibody anti-AGP 1:2000 (rabbit two); secondary antibody: peroxidate anti-rabbit 1:2000. D) First lane: 100 ng of pure goat AGP, second lane: 1µl of goat serum; primary antibody anti-AGP 1:2000 (rabbit two); secondary antibody: peroxidate anti-rabbit 1:2000.Detection by chemiluminescence (ECL, Millipore).

The experimental design applied to develop an ELISA protocol to detect goat AGP was similar to that carried out for the development of the test in cattle Bovine AGP was used as standard. The antibody produced by rabbit 2 (poly AGP2) was used as coating antibody at an amount of 250 ng per well. Antibody polyAGP1 was used as the

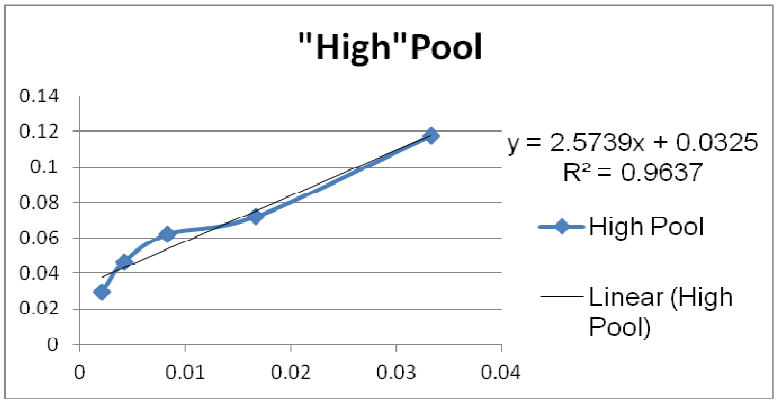
biotynilated one, to identify the proteins, at the same concentration of poly AGP2.The standard curve used was the same of bovine assay. In the several tests carried out the ideal dilution of samples of goat plasma was determined as 1:8, since at this dilution the absorbance values that are obtained falled in the middle of calibration curve.

As described before, to validate the test it was necessary to create a pool with sera of healthy animals, in order to obtain a pool with low values of AGP. Pooled sera of diseased animals were obtained as well.

The analytical validation of the assay was carried out as previously described.

Assessment of precision. The CV of the serum pool for high values of AGP was of was of 15.6 % (INTER-assay). The serum pool with low values of AGP presented a CV of 16.3 %. AGP CV of INTRA-assay was 15.4 % for "high" pool and 15 % for "low" pool.

Assessment of the accuracy. The R^2 for the "high" pool was of 0.9637, whereas for the 'low' pool was of 0.9774. Both these values confirm that the test has an acceptable accuracy, since both are close to one. For what concerns the recovery data obtained, values forboth the "high" and "low" pool were close to 100 % indicating that the test has a good accuracy.



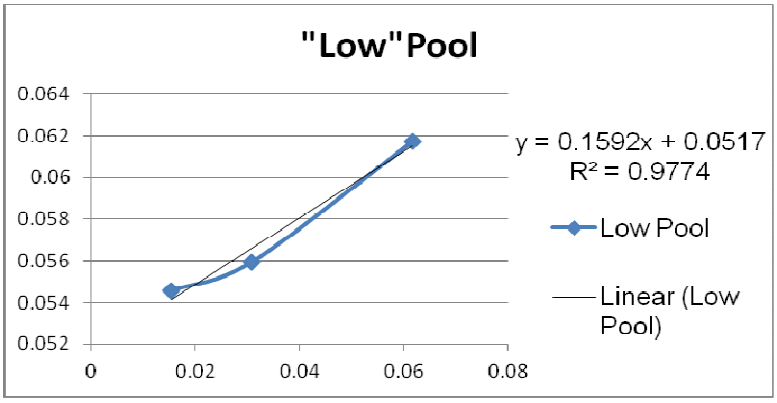


Fig.6.4.3.6: graphic representation of "High" pool and "Low" pool linearity in goat test.

Evaluation of sensitivity. For this assay the limit of detection that is equal to 0.062 µg/ml.

Finally, also for this test ULOQ and LLOQ were calculated, (2.5 µg/ml and 0.062 µg/ml respectively).

The ability of the assay to distinguish between healthy and diseased animals was tested in 22 serum from healthy goats and 68 from goats with disease (17 with brucellosis, 16

with CAEV and 35 with intramammary experimental infection by *E. coli*) were analyzed (Fig. 6.4.3.7).

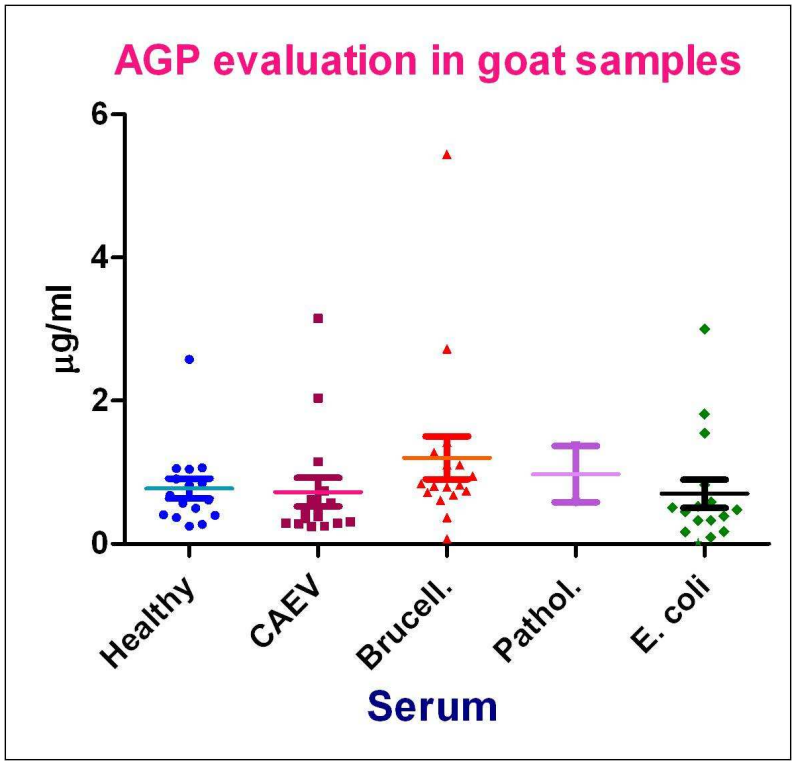


Fig.6.4.3.7: graphic representation of goat serum evaluation.

In our experiments, no statistically significant differences in the levels of AGP between healthy animals and animals with diseases were detected with the ELISA test which was developed during this thesis. However it is noticeable that AGP levels in samples with brucellosis are someway higher than the others.

6.4.4 DISCUSSION

In this part of the thesis we investigated the possibility that this protein could be used as a biomarker to early identify a disease not yet clinically evident. In the present study, a precise and sensitive test based on Enzyme-Linked Immuno Sorbent Assay is described, which was useful to determine AGP content in serum of bovine and goats. We have validated our ELISA based on the standard criteria of precision, accuracy and analytical sensitivity. The linearity studied indicated that our method detects the amount of protein present in a linear and proportional way. Our ELISA demonstrates good analytical sensitivity and is capable of detecting a wide range of AGP concentrations in bovine and goat serum. Yet, neither in goats nor in bovine species, the test managed to identify the animals with disease. The test is apparently working on healthy animals, and therefore we may speculate that the concentration of AGP in animals with diseases is not increased in a statistically significant way. AGP is not a major acute phase protein in the two species included in this experiment *(Ceciliani et al., 2012)*, and therefore it is also possible that the concentration of AGP does not increase for this simple reason. Results that we have obtained in bovine assay confirm what previously published. Indeed Orro *(Orro et al., 2008)* and colleagues demonstrated that temporal changes occurred in bovine APPs within the first 3 weeks of life: all APPs after birth were higher than in adults, but then show a decrease. The changes are most pronounced in SAA and AGP concentrations, and, in general, concentrations were higher within 2 weeks of birth than later, in all proteins. Stabilization of APP concentrations occurred after 3 weeks of age *(Orro et al., 2008)*. Differences in neonatal and adult AGP isoforms *(Itoh et al., 1993)* and a rise in AGP concentration already in fetal stages have been reported in calves and

piglets *(Stone and Maurer, 1987* and *Itoh et al., 1993)*. This indicates that neonatal AGP is probably fetally regulated. High neonatal serum concentration of AGP is not necessarily related to the activation of APR by some external stimulus, which is supported by the continued gradual decrease after birth.

It has been shown that AGP does not increase in some diseases, such as for example in CAEV, but changes its post-translational modification, which also could, anyway, modify also its interaction with antibody *(Ceciliani et al., 2012)* and therefore possibly decrease the sensitivity of the test. Yet, in brucellosis for example, an increased, albeit statistically not significant concentration of AGP was detected. It is also possible that by increasing the number of pathological samples a statistical positive result might be obtained.

It must also be said that the current AGP "golden standard" in veterinary medicine is a Radial Immuno Diffusion bas assay, whose sensitivity is 50 μg/ml. The sensitivity obtained in the ELISA assay is more that three order of magnitude more sensitive (0.03 μg/ml) than the actual one.

6.4.5 CONCLUSION

This study developed a novel sandwich ELISA for measuring AGP in bovine and goat species. The assay is suitable for use in healthy animals. More "unhealthy samples" must be analyzed in order to achieve a clinical validation of the assay. The increased sensitivity of this new ELISA will greatly facilitate the ongoing investigation of the role of AGP in the APR in cattle and goats.

6.5 Production of polyclonal antibody anti-bovine SAA in rabbit and development of an ELISA test for the quantification of SAA in bovine and goat serum.

6.5.1 OBJECTIVES AND EXPERIEMNTAL DESIGN

The aim of the present study was to develop of an ELISA test for the quantification of Serum Amyloid A (SAA) in cattle and goat serum.

The study was divided into two parts:

- immunization of rabbits with specific peptide (MAP) for the stimulation of the immune system and therefore production of antibody;
- development of ELISA test for the quantification of SAA.

6.5.2 INTRODUCTION: THE MAP STRATEGY

Because of technical difficulties, in particular because it is very difficult to purify SAA from healthy animals, and SAA purified from unhealthy ones is unstable and precipitates, no species-specific assays have been developed to measure this protein in bovine and goats serum to date.

The rationale behind this experiment was to identify a short peptide that could be synthesized, and use it as standard. The strategy was to synthesize a Multiple Antigen

Peptide (MAP), to produce specific antibodies against bovine SAA, because the obtaining of Ab which allows recognizing the SAA in a specific manner was found to be an extremely complex process.

Synthetic immunogenic peptides are ideal vaccine subunit components because of the following differences to traditional vaccines composed whole proteins. However, the biological activity of peptides is generally short due to enzymatic degradation, and small peptides that are used as antigens are not recognized by immune cells, e.g., dendritic cells (DCs) and macrophages, and do not elicit a strong immune response when administered alone. The co-administration of adjuvants (e.g., water-in-oil emulsions, oil-in-water emulsions, liposomes, bacterial lipophilic compounds, etc.) with subunit peptide antigens is one of the methods used to enhance the immune response. Alternatively, short antigenic peptides induce strong immune responses when co-administrated or engaged with carrier proteins (e.g., ovalbumin (OVA), bovine serum albumin, keyhole limpet hemocyanin, tetanus toxoid, etc.); however, they are also associated with undesirable effects such as the suppression of the anti-peptide Ab response and the production of Abs against the carrier proteins *(Herzenberg et al., 1980; Landsteiner, 1962; Avery and Goebel, 1931; Schutze et al., 1985; DiJohn et al., 1989).*

Tam developed the multiple antigenic peptide (MAP) system to improve the poor immunogenicity of subunit peptide vaccines *(Tam, 1988).* In this MAP system, multiple copies of antigenic peptides are simultaneously bound to the α- and ε-amino groups of a non-immunogenic Lys-based dendritic scaffold (Figure 6.5.2.1).

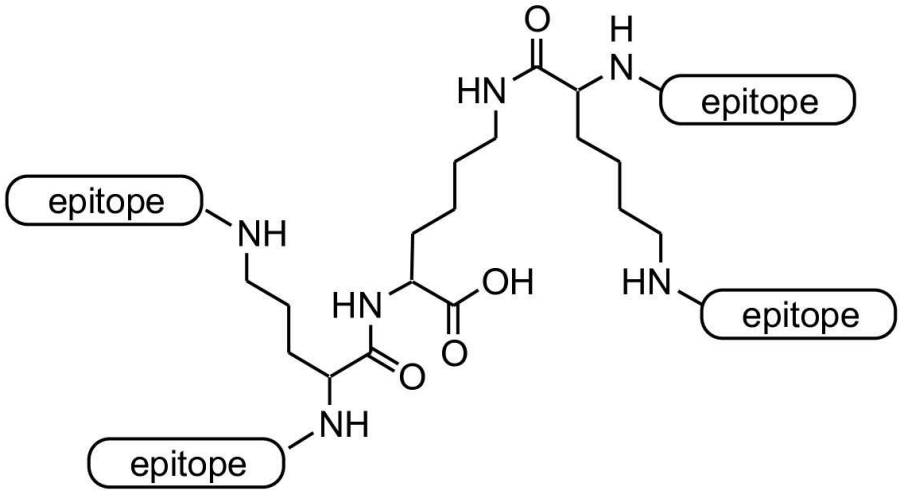


Fig. 6.5.2.1: Lys-based MAP vaccines. *(Fujita and Taguchi, 2011).*

The protein-sized MAP molecules acquire stability from enzymatic degradation, enhanced molecular recognition by immune cells, and induction of stronger immune responses compared with small antigenic peptides. The MAP concept represented a major breakthrough for immunization systems and further improvements in a large variety of multiple antigens-presenting peptide vaccine systems have been investigated.

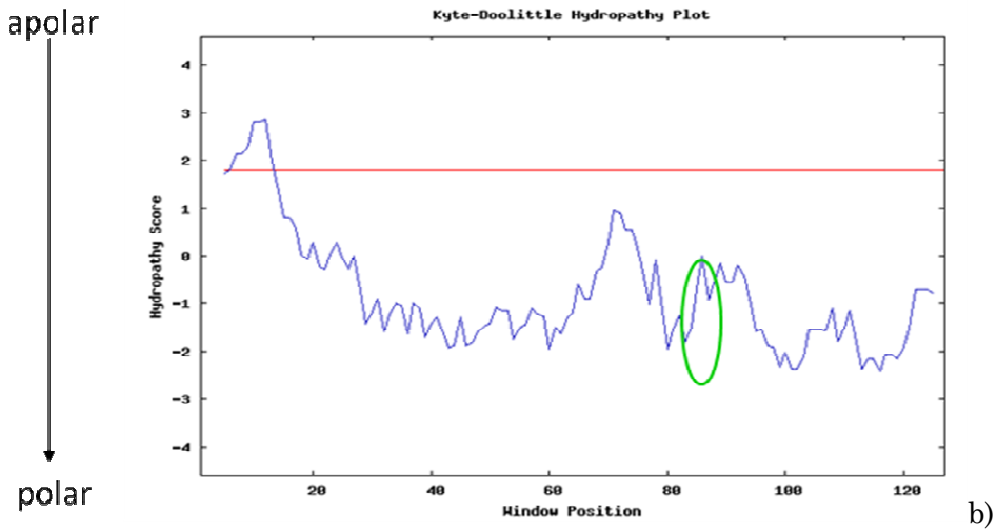
The utilization of a peptide also overcame low solubility of SAA that drives the protein to precipitate. For all these reasons it was designed a MAP with the aim to obtain specific Ab for the recognition of bovine SAA. A comparison analyses between animal known sequences was carried out, and a short peptide unique for ruminants was identified, differing from SAA rabbit. A simulation of hydropathy of SAA identified the peptide within a hydrophilic domain. Therefore, following these in silico analysis, the peptide was identified as a possible immunogen peptide for anti-SAA antibodies, as shown in Figure 6.5.2.2.

```
sp|P35541|SAA_BOVIN     MKLFTGLILCSLVLGVHSQ-WMSFFGEAYEGAKDMWRAYSDMREANYKGADKYFHARGNY 59
sp|P22000|SAA2_RABIT    MKLLSGLLLCSLVLGVSGQGWFSFIGEAVRGAGDMWRAYSDMREANYINADKYFHARGNY 60
                        ***:**:******** .* *:**:*** .** ************** .**********

sp|P35541|SAA_BOVIN     DAAQRGPGGAWAAKVISDARENIQRFTDPLFKGTTSGGGQEDSRADQAANEWGRSGKDPN 119
sp|P22000|SAA2_RABIT    DAAQRGPGGVWAAKVISDVREDLQRL---------MGHGAEDSMADQAANEWGRSGKDPN 111
                        ********.********.**:**.           *.* *** ****************

sp|P35541|SAA_BOVIN     HFRPAGLPDKY 130
sp|P22000|SAA2_RABIT    HFRPKGLPDKY 122
                        **** ******
```

a)



b)

Fig. 6.5.2.2: a) Sequence fragment of bovine SAA on which has been drawn the MAP. This sequence, apart from to differ from that of rabbit, was chosen because it was the one that had a greater hydrophilicity (essential characteristic for the inoculum) b).

The objective of this part of the experiment was to develop a novel immunoassay to measure SAA in bovine and goats serum. Polyclonal antibodies were produced in rabbits immunized with MAP. MAP and polyclonal antibodies were then used in an ELISA sandwich assay.

6.5.3 MATERIAL AND METHODS

Production of rabbit polyclonal antibodies

The protocol that we used to produce antibodies able to detect SAA is the same operated to obtain the polyclonal antibodies against AGP. In this case, rabbits were immunized with 2.5 mg/ml of pure Multiple Antigen Peptide (MAP).

At the same time, we used the protocol described at page 114 to obtain an aliquot of antibodies labeled with biotin.

Western blot analysis

Samples tested were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotted onto nitrocellulose membranes. The membranes were immunolabelled for the presence of SAA using the specific antibodies (that we had produced) and immunoreactive bands were visualised by enhanced chemiluminescence (ECL) using Immobilon Western Chemiluminescence substrate (Millipore).

Enzyme-linked immunosorbent assay (ELISA)

Instrumentation and Materials

The instrumentation and materials that we used for this assay are the same that we utilized for ELISA AGP, illustrated at page 115.

The development, optimization and validation of this assay were divided in several steps:

1.~Selection of the suitable antibody for the competitive ELISA assay: the response of each of the two different antibodies to a competitive assay was assessed. In brief, Polystyrene 96 wells microtiter plates (Nunc-Immunoplate Maxisorp, Nunc, Denmark) were coated over-night at 4 °C with 100 µl/well of a specific bovine polyclonal antibody (0.5 µg/ml) in carbonate/bicarbonate buffer, pH 9.6. Plates were washed four times in phosphate buffer saline containing 0.1 % (v/v) Tween-20 (PBS-T). 250 µl/well of blocking solution RotiBlock 1X (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were dispensed and the plates were incubated over-night at 4 °C. Subsequently, plates were washed four times in phosphate buffer saline containing 0.1 % (v/v) Tween-20 (PBS-T). One hundred microliter of a series of double dilutions standard (pure MAP) solution ranging from 10 to 0.15625 µg/ml per well was added to one row along with dilution buffer (RotiBlock 1X), sample (conveniently diluted in blocking solution 1X) and blank, were added in duplicate to the appropriate well, and the plates were incubated 1 h at 37°C. Afterward, plates were washed four times in phosphate buffer saline containing 0.1 % (v/v) Tween-20. One hundred microliter of a specific rabbit polyclonal biotinylated antibody (0.5 µg/ml) opportunely diluted in RotiBlock 1X were dispensed and the plates were incubated 1 h at room temperature in a flutter. After washing, 1:1000 streptavidin-perxidase polymer-ultrasensitive (Sigma-Aldrich Corp., St. Louis, MO, USA) was incubated for 1 hour at room temperature in a flutter in the dark and the reaction was measured by the addition of ABTS substrate (Sigma-Aldrich Corp., St. Louis, MO, USA) and 405 nm kinetic reading every 5 minutes for 20-60 minutes in a microplate spectrophotometer reader (Labsystem Multiskan MS).

2.~Optimization of antibody and biotinilated antibody concentrations: 50, 100, 150 175, 250 and 375 ng of antibody per well, in combination with standard curve obtained with

MAP, and 50, 100, 150 175, 250 and 375 ng of biotinilated antibody per well were tested. The combination that combined both minimum concentration of reactants and good regression fit was chosen.

3.~Standard curve: Several dilutions of MAP were tested to build a standard curve that would allow a wide assay range and avoid hook effects, showing a good fit to regression analysis.

4.~Optimal sample dilution: a range between 1:2 to 1:128 of two bovine sera pools (or two goat sera pools), one with presumably high and one with presumably low concentration of SAA were tested to determine the concentration than best fitted the range of the standard curve. Each pool was prepared by mixing serum samples from different animals with supposedly similar SAA concentrations.

Statistical Analysis

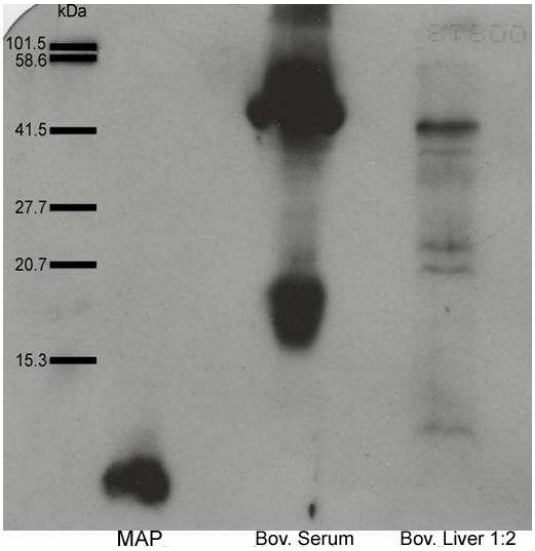
Regression analysis and One Site Total non-parametric test was used by the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com"). t Test of significance (significance level was settled at $p < 0.05$) was used to compare values of SAA in healthy and diseased bovine, or goat, were carried out by standard descriptive statistical methods by using Microsoft Excel 2000 (Microsoft Corporation. USA).

6.5.4 RESULTS

Bovine assay

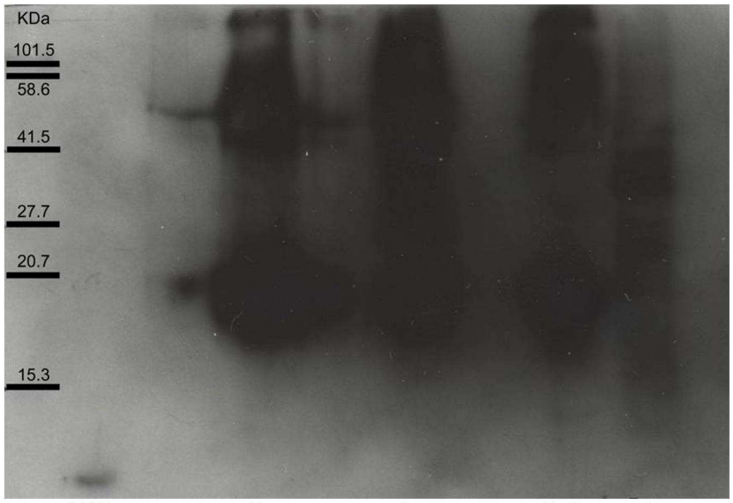
Western blot analysis

In the first part of this study we obtained two antibodies that can specifically recognize bovine SAA (Fig.6.5.4.1).



a)

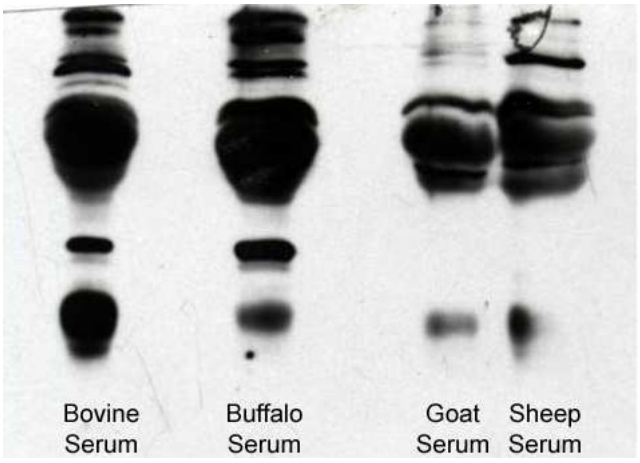
Fig. 6.5.4.1: a) Western Blot for analyze the specificity of antibodies. First lane: 5 ng of pure MAP; second lane: 1µl of bovine serum; third lane: proteins extract from bovine liver diluted 1:2; primary antibody polySAA1 1:500; secondary antibody: peroxidate anti-rabbit 1:2000. Detection by chemiluminescence (ECL, Millipore).



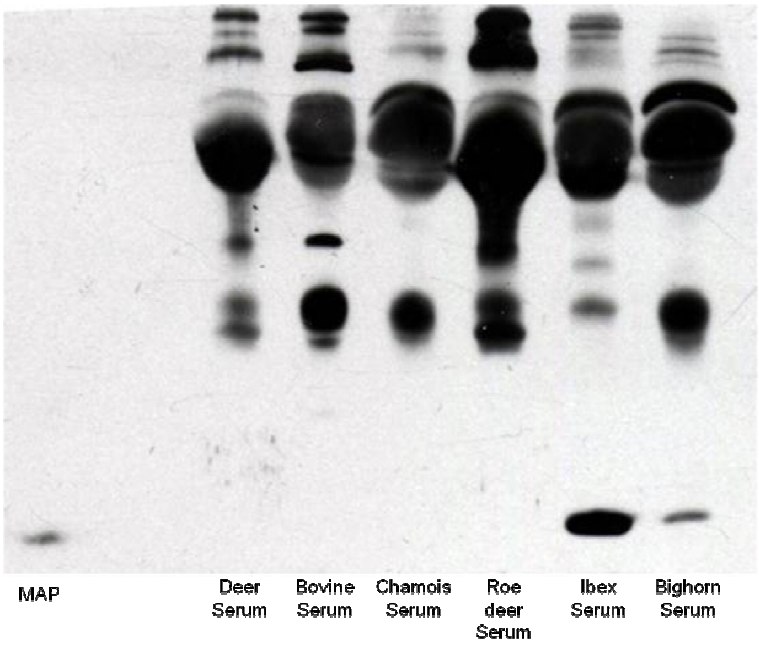
b)

Fig. 6.5.4.1: b) Western Blot for analyze the specificity of antibodies. First lane: 5 ng of pure MAP; second lane: 1µl of bovine serum; third lane: 1µl of goat serum; fourth lane: 1µl of buffalo serum; fifth lane: proteins extract from bovine liver diluted 1:2; primary antibody polySAA2 1:400; secondary antibody: peroxidate anti-rabbit 1:2000. Detection by chemiluminescence (ECL, Millipore).

By Western Blot analysis is evident that the antibody produced from rabbit 1 is able to provide the best answer. PolySAA2 can still recognize the protein, but the high background prevents its utilization as detection antibody. PolySAA1 was also tested for its cross-reactivity in other species of ruminants (both domestic and wild) (Fig. 6.5.4.2).



a)



b)

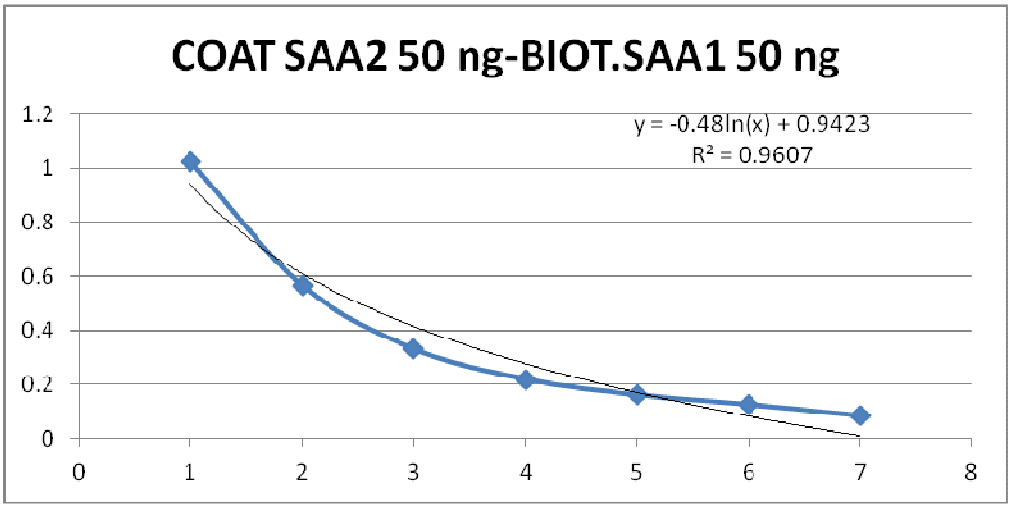
Fig. 6.5.4.2: Western Blot to analyze the cross-reactivity of antibody product by rabbit one in different ruminant species. In each lane it was loaded 1µl of serum for each species. a) Western Blot analyzing on domestic ruminant; b) Western Blot analyzing on wild ruminants.

ELISA analysis

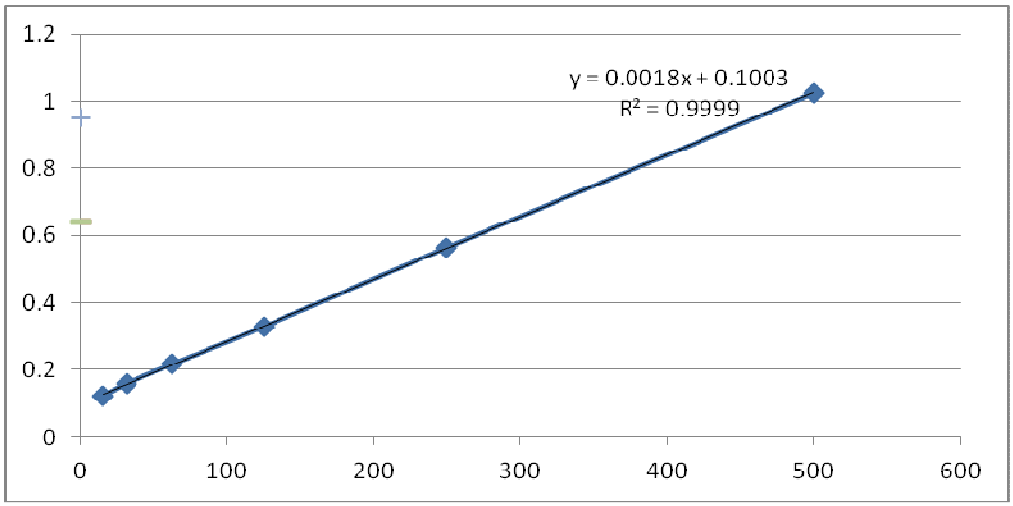
Goat assay

Although, in the Western Blot analysis the antibody produced by the rabbit two has not been able to give a good response when compared to that produced by the rabbit one, we decide to develop ELISA test trying both antibodies, since it is known that immunoreactivity with Western Blotted proteins (which are denatured) and soluble proteins (which are in native conformation) may be different. For the test ELISA to detect goat SAA the best antibodies concentration to use as coating was identified to be 50 ng per well and the antibody used was the one produced in rabbit 2; for biotinylated antibody the right concentration was identified to be the same of coating, and polySAA1 antibody was used.

Regarding the calibration curve, MAP peptide was used, starting from a peptide concentration of 10 µg/ml up to 0.15625 µg/ml, in order to assess the concentration of SAA in an accurate manner without encountering the so-called "hook effect". The optimal dilution of samples of goat plasma was shown to be to be 1:4, since at this dilution the absorbance values that are obtained falled in the middle of standard curve.



a)



b)

Fig. 6.5.4.3: the calibration curve, constructed using MAP using the following concentrations of protein: 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15626 µg/ml. a) logarithmic scale; b) linear scale.

The capability of the assay to distinguish between healthy and diseased animals was tested in serum from healthy goats and from goats with disease.

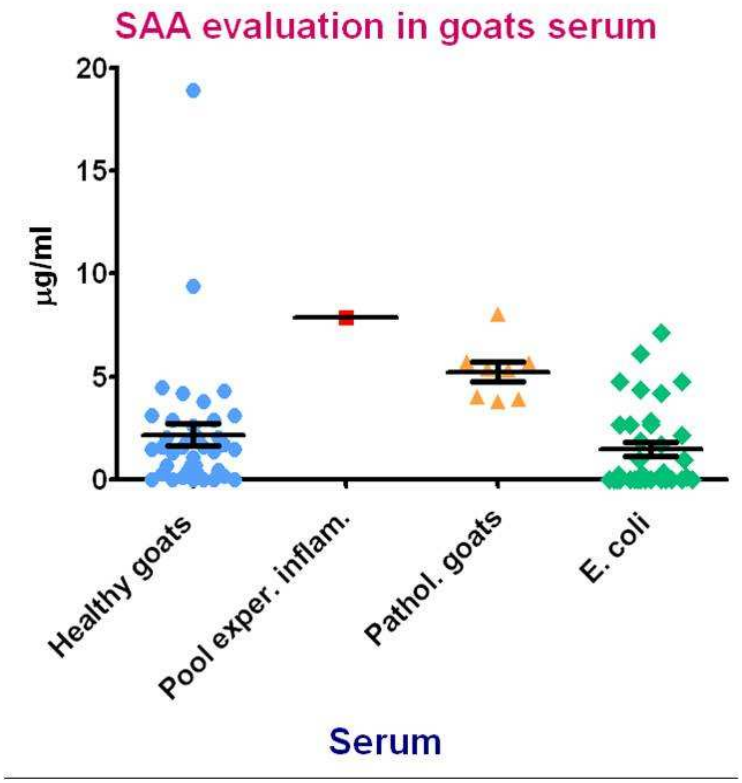


Fig. 6.5.4.5: graphic representation of goat serum evaluation.

In our experiments, no statistically significant differences in the levels of SAA between healthy animals and animals with diseases were detected in a statistically different way. However it is noticeable that SAA levels in pathological pool (made by 20 different sera of goats experimentally injected with Turpentine oil) is higher than the others. Results from pathological goats are someway higher as well.

Bovine assay

For the test ELISA to detect bovine SAA we continued with the same steps carried out for the development of the test in goats. Given the limited amount of assays, the results obtained in this section should be considered extremely preliminary.

Regarding the standard curve it was used the same of goat assay obtained with the MAP. In the various tests carried out is also seen that the ideal dilution of samples of bovine serum resulting to be 1:2, since at this dilution the absorbance values that are obtained fall in the middle of calibration curve.

The ability of the assay to distinguish between healthy and diseased animals was tested in serum from healthy bovine and from bovine with disease.

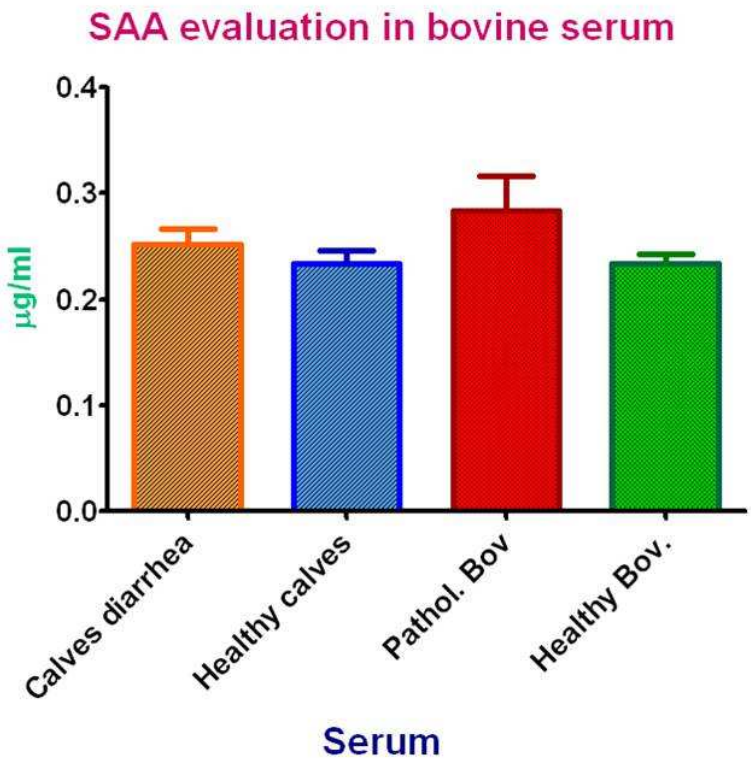


Fig. 6.5.6 graphic representation of bovine sera evaluation.

In our studies, no statistically significant differences in the levels of SAA between healthy calves and calves with diarrhea were detected with our ELISA test, and the same results were observed between healthy bovine and bovine with disease.

6.5.5 DISCUSSION

Biochemical investigations have central function in clinical chemistry with an important aid to provide principal biochemical information for clinicians in the diagnosis, prognosis, monitoring, and screening of diseases.

The reason for developing accurate methods to measure SAA in ruminant is its potential use in monitoring their health and welfare and in assessing the quality of their products *(Eckersall et al., 1999)*. SAA is particularly interesting in this context as it exhibits a transient but intense response to a range of inflammatory stimuli *(Sorensen et al., 2006)*. Moreover, SAA is the main acute phase protein in ruminants.

The use of serum amyloid A has been limited due to difficulties in purification and quantification, probably because it is a hydrophobic apolipoprotein that is complexed within serum high-density lipoproteins *(Yamamoto et al., 1994)*.

By using the MAP we circumvented the problem of purifying SAA from serum, a laborious, low-throughput method that often results in low yields or impure or insoluble product.

The results of this part of the thesis must be regarded as preliminary. The very limited number of cows analyzed prevents to drive any conclusion. The animals were not available in a statistically significant number, and therefore these analyses must be repeated for both healthy animals, in order to reach a number sufficient for analytical validation and unhealthy, in order to get sufficient samples for clinical validation.

For what concerns goats, then number of samples must be increased as well. While statistically non significant, the increase of SAA concentration in unhealthy goats is encouraging, and therefore further experiments to complete this experiment are strongly envisaged.

One weak point of the present anti-SAA ELISA protocol was the low dilution of the samples. While the assay provides a good sensitivity, which is comparable to that of other available ELISA assays (Tridelta ltd), yet it is not possible to dilute too much the samples, making the assay quite “expensive” from a sampling perspective.

6.5.6 CONCLUSION

In this study, we have developed a novel immunoassay to detect levels of SAA in ruminants' serum, in particular our test it was focalized on SAA's levels of bovine and goats. The next step of this study will be obtain the analytical and clinical validation of the test. For this we must collect a right number of sera that display high levels of SAA and low levels of this protein to obtain “high” and “low” pool. Then, we could analyze a large number of healthy and pathological samples to demonstrate statistically significant or no statistically significant differences between sera.

7. Final remarks and possible developments

The aim of this research project carried out in the three years of PhD was to identify possible biomarkers to help to detect rapidly, timely, and economically conveniently, the presence of any diseases clinically not evident. These biomarkers were evaluated in ruminant species.

In particular, during the first year I have worked to answer the question about where APP are coming from, assessing their presence (and quantifying mRNA expression) from bovine forestomachs and, more in general, from all other tissues. This concept is quite new, since the activity of protein secretion of the forestomach was almost unknown.

My study, in the first place, has demonstrated that the mucosa of the forestomachs are able to produce RNA of proteins destined to be secreted, showing that the major acute phase proteins are produced locally and not only in the liver. The forestomachs' mucosa is a surface constantly in contact with microbial agents, so it is directly involved in the control of local immune response and in the maintenance of homeostasis of the organism and it can be speculated that it is precisely for this reason that there is a production of APP in these tissues. In conclusion, the high expression at the level of the mucosal surface of the forestomach and abomasum supports the hypothesis that the acute phase proteins play a role of primary importance in modulation of the innate immune response and local homeostasis of the organism, and identifies forestomachs as a source of potential biomarkers for disease.

A second objective targeted was that to extend the study of the expression of SAA and Hp in 33 different tissues of two healthy cattle. In order to investigate the mRNA

expression of these two proteins, the first step was to find a set of reference genes to normalize the data obtained from the Real-Time PCR. The relative quantification of SAA and Hp was performed after normalization with three selected genes. It was interesting to note how the SAA and Hp have been identified in tissues of healthy cattle also in the absence of a systemic reaction of the acute phase.

In particular, the SAA and Hp, are highly expressed in organs communicating with the external environment, such as the mammary gland, lungs or salivary glands. These data may mean that SAA and Hp constitute a part of a host defense mechanism involved in protection of these tissues from the attack of microorganisms and possible injury, and also support further experiments to assess the presence of these proteins in biological fluids other than serum, such as milk, or saliva.

A third topic that was investigated was the capability of $CD14^+$ cells to produce miRNA after stimulation with Pathogen Associate Molecules. This experiment is preliminary to the assessment of miRNA as possible biomarkers in bovine species. Bovine $CD14^+$ monocytes can produce all the considered miRNAs. Secondly we observed that a few miRNAs change their expression in stimulated cells compared with non-stimulated cells. The future perspective of this experiment may be the identification of a number of other cattle miRNAs whose expression changes in different ways according to the pathology, in order to use them as markers for diagnosis. The application of miRNA as biomarkers is becoming of growing importance in human medicine, and it cannot be ruled out a future extensive utilization in veterinary medicine as well.

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The last step of this PhD was devoted to the developing of protocols to assess by ELISA the concentration of two acute phase protein in ruminants, SAA and AGP. The reason for developing accurate methods to measure AGP and SAA in ruminant is their potential use in monitoring animals' health and welfare and in assessing the quality of their products. While the results of this last part of the thesis are encouraging, since we have analytically validated AGP ELISA, still the data that I presented must be regarded as preliminary, and must be confirmed by analysis on more animals.

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